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Studying the Effects of Theraflu on the Growth of Selenastrum Capricornutum

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I. Introduction

Algae contribute to self-purification of streams and rivers and are necessary as food for fish and as components of aquatic food webs (1). However, too much or too little algae may create or be indicative of a problem. If nutrients are present in large amounts, algae growth may become excessive, resulting in algal "blooms." These algal blooms can change the chemistry of the water, making it toxic to other aquatic occupants, including fish, birds, animals, and other plants (1). On the other hand, if nutrients in the water are limiting or are exhausted, algae growth is inhibited, which results in lower oxygen production via photosynthesis (1). In other words, the composition and abundance of algae directly affect water quality. In addition, algae composition and abundance is a direct result of the availability of nutrients. In order to compare algal growth potentials from a number of widely different water sources there are advantages in using a single representative species of alga. It is ideal for the alga being used to be readily available and for its growth to be measured easily yet accurately (1).

For this study, the freshwater green alga *Selenastrum capricornutum* was used as a model for the aquatic environment. *S. capricornutum* was first isolated in 1959 by O.M. Skulkberg in the River Nitelva in Akershus, Norway (4). The taxonomy of *S. capricornutum* is as follows:

Domain: *Eukaryota*
Kingdom: *Plantae*
Phylum: *Chlorophyta*
Class: *Chlorophyceae*
Order: *Sphaeropleales*

Family: *Selenastraceae*
Genus: *Selenastrum*
Species: *capricornutum*

S. capricornutum is a crescent-shaped freshwater green alga which has a single chloroplast found within the algal cell and occasionally forms non-mucilaginous colonies of four to sixteen cells. These colonies are rarely intertwined and, instead, form a matrix by attaching their dorsal sides to other cells.

S. capricornutum is important in bioassays of water quality and environmental assessment (2). It is a sensitive species and is one of the three chosen organisms by the Environmental Protection Agency as a toxicity test species in the National Pollutant Discharge Elimination System (NPDES). Algae have long been used in water quality assessment as *in situ* bio-monitors, but have not been commonly used in toxicity tests (6). Therefore, a small toxicity database exists which is not consistent with the ecological importance of algae in aquatic ecosystems (6). In response to this, Terry W. Snell, Joy L. Mitchell, and Susan E. Burbank performed a study that focused on rapid toxicity assessment in four different species of algae (6). This study tested seven different toxicants: pentachlorophenol, naphthol, chlorpyrifos, cadmium, mercury, copper, and phenol. The four algae species tested were *Selenatrum capricornutum, Tetraselmis suecica, Cyclotella* sp., and *Synechococcus leopoliensis*. In the study, *S. capricornutum* was the most sensitive alga used for five of the seven toxicants (6).

My study involves studying the effect of Pharmaceuticals and Primary Care Products (PPCPs) on water quality in the environment. PPCPs can be drugs, soaps,

lotions, and other such compounds. These PPCPs affect the environment when they enter water systems via, flushing the toilet and human and animal excretions. The question that this study tries to address is how these seemingly harmless drugs affect our environment and water quality.

The PPCP being studied is the over-the-counter drug Theraflu, containing diphenhydramine HCl, acetaminophen, and phenylephrine HCl. These three drugs are the active ingredients in several nighttime cold and allergy medicines, e.g. Sudafed, Delsym, Theraflu (5). Diphenhydramine HCl is an active ingredient in most sleep aids and antihistamines, particularly Benadryl and ZzzQuil (7). Theraflu was chosen to study based on its widespread popularity and its availability in liquid form.

S. capricornutum functions in this study as a test species for the aquatic environment. The hypothesis is that if the growth of *S. capricornutum* is inhibited, then it is possible that a component of Theraflu—diphenhydramine HCl, acetaminophen, or phenylephrine—affects the environment negatively.

II. Methods

For this experiment the procedure found in the *Algal Assay Procedure Bottle Test* was followed (3).

Cleaning the Glassware

To begin a trial, it is necessary to clean the glassware being used. As described in the *Algal Assay Procedure Bottle Test*, all cylinders, flasks, bottles, centrifuge tubes and vials were washed with detergent and rinsed thoroughly with

tap water. This was followed by a rinse with ten percent HCl solution. All containers are filled to approximately one-tenth capacity with HCl solution and swirled so that the entire inner surface is bathed. After the HCl rinse, the glassware was rinsed eight times with deionized water (3).

Preparing the Media

After preparing the glassware, the medium was prepared. The experiment used 250 mL of medium in 500 mL Erlenmeyer flasks. This medium contained nutrients that allow the algae to grow. The composition of the medium can be found in the *Algal Assay Procedure Bottle Test* (3). Once the medium was dispensed into the flasks, an autoclave was used to sterilize the medium. Four flasks containing the medium were autoclaved at 15 psi for 15 minutes at 121° C. After autoclaving and cooling, the sample was allowed to equilibrate in an air atmosphere in order to restore the carbon dioxide lost during autoclaving and to lower the pH to its original level.

Treatment Flasks

For this study, Wal-flu was used as a cheaper, but comparable alternative to the Theraflu. For the remainder of this paper, the Wal-flu will be referred to as Theraflu. Four flasks were used in each trial of the experiment. One flask served as a control containing none of the experimental compound and three flasks served as treatment samples containing increasing concentrations of Theraflu. In one of the treatment flasks, 200 μL of Theraflu—containing diphenhydramine HCl, phenylephrine HCl, and acetaminophen—was added to the 250 mL media. For this study, 200 μL was designated as the 1X flask. In a second flask, 400 μL of the

combination drug was added. This flask was labeled the 2X flask. In a third flask, 800 µL of the combination drug was added, and this flask was labeled the 3X flask. In a fourth flask, there was no drug added and this flask was labeled the control flask. Into each flask, five mL of *S. capricornutum* was added from the previous week's control flask.

Table 1: Contents of each flask in each combination drug trial

	Control	1X	2X	3X
Amount of combination drug (Theraflu)	0 µL	200 µL	400 µL	800 µL
Amount of algae (*S. capricornutum)*	5 mL	5 mL	5 mL	5 mL

The flasks with the cultures added were set under continuous cool-white fluorescent lighting at a temperature of 24 °C. They were stirred continuously to ensure that there was sufficient gas exchange. Each culture was allowed to grow for one week and then the number of cells in each was counted.

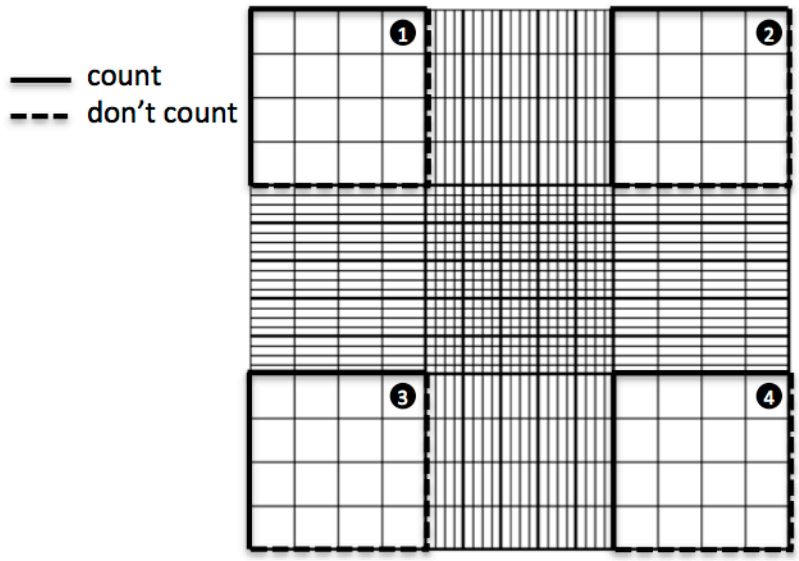
When the flasks were removed from the stirrers, the contents of each flask were transferred to an appropriately labeled cylindrical flask. The samples were then placed under a cardboard box in a 4 °C refrigerator. This created a cool, dark environment in which changes in the samples were minimal.

Determining Cell Density

To determine the number of cells/mL, we used a hemocytometer. Each sample was diluted with Milli-Q water according to how green the algae looked; the

greener the algae, the more concentrated the cells. If the algae looked fairly green, a one-fourth dilution was used; one-fourth meaning one-part algae and three parts Milli-Q water. If the sample looked only slightly green, a two-part dilution was used (one part algae, one part Milli-Q water).

A hemocytometer is a square chamber carved into a piece of thick glass that has a specific depth and holds 10 µL. This big square has other little squares inside, following the pattern below (9).



Using a counter, the number of cells was counted in each of the four corner squares outlined in bold. Because cells can only be counted once, and some cells lay half-in half-out the square being counted, two sides of each square were chosen to count cells that were touching the line and on the other two sides of each square the cells that were touching were not counted. After counting these four squares, an average number of cells were calculated for each sample.

Using this average, I calculated the cell density using the following formula:

$$\frac{cells}{mL} = (Avg.\#\ of\ cells/\mu L)(dilution)(10{,}000\ \mu L/mL)$$

After doing the cell counts for each flask of the trial, a graph was constructed for analysis. If the hypothesis were correct, there should have been a negative correlation between the cell density and the drug concentration. As drug concentration increased, cell density was expected to decrease.

Individual drugs

After conducting several trials with Theraflu containing the three drugs in combination, acetaminophen and diphenhydramine HCl were each tested separately on the algae. Phenylephrine HCl was not found in an over-the-counter drug by itself. Therefore, it was not tested.

Acetaminophen

The acetaminophen used was in the form of a tablet. This called for the tablet to be ground up with a mortar and pestle and put into solution in order that it could be used. To begin, we calculated how much acetaminophen should be put into the solution so that it was equivalent to the amount in the combination drug, Theraflu. The combination drug used in the six trials above contained 325 mg of acetaminophen per 15 mL, i.e. 21.67 mg/mL acetaminophen in Theraflu. Starting with the amount of Theraflu and the drug concentration of acetaminophen in Theraflu used in the previous treatment flasks, the amount of acetaminophen in each flask was calculated.

$$0.2\ mL \times 21.67\frac{mg}{mL} = 4.3\ mg\ of\ acetaminophen\ in\ Theraflu\ 1X\ treatment\ flask$$

The acetaminophen tablets were 325 mg each. In order to put it into solution, two tablets (650 mg) were dissolved into 500 mL of water. This amounted to 1.3 mg/mL. Next, the amount of acetaminophen needed to put into the treatment flasks was in each of our 1X, 2X, and 3X samples used with the combination drug. From this number, a calculation was done to figure out the equivalent amount of acetaminophen to use in their separate trials.

For example, in the 1X sample the volume of acetaminophen solution that should be used was calculated in order to have equivalent amounts of acetaminophen in the separate, acetaminophen only treatment.

$$4.3\ mg\ of\ acetaminophen\ in\ Theraflu\ 1X$$

$$\approx 1.3 \frac{mg}{mL}\ acetaminophen\ individually \times \mathbf{4\ mL}\ of\ solution$$

To create a 1X flask that contained equivalent amounts of acetaminophen as used in the previous trials, 4 mL of the acetaminophen solution was used. Using the same method as above, 6 mL of acetaminophen solution was calculated to equal the 2X Theraflu flask. To create 3X flask that contained equivalent amounts of acetaminophen as used in the previous trials, 14 mL of the acetaminophen solution was used to test the algae.

Table 2: Contents of Flasks in Acetaminophen only trial

	Control	1X	2X	3X
Acetaminophen 1.3 mg/mL conc.	0 mL of conc.	4 mL of solution	6 mL of solution	14 mL of solution
Algae	5 mL	5 mL	5 mL	5 mL

Once the acetaminophen was placed in 500 mL of water, if it did not dissolve the solution would be placed over heat to try to dissolve the solution. If it still did not dissolve after heating, then it is recommended that the solution be shaken well before taking any out to put in the flasks. If the suspended cell counts turned out low, it could possibly be because the algal cells were clumping around the undissolved acetaminophen. Algal cells are very "sticky." They will easily clump around any particles present in the media whether this is bacteria, undissolved drug, etc.

Diphenhydramine HCl

The same calculations were done with the individual drug diphenhydramine HCl, except this drug was available in liquid form. The drug used for this was Wal-Sleep Z, the Walgreen's equivalent of Nyquil. The availability of this drug in liquid form eliminated the need for a mortar and pestle and solution. In the combination drug, there is 12.5 mg of diphenhydramine HCl per 15 mL, i. e. 0.83 mg/mL. In the Wal-Sleep Z, there is 50 mg of diphenhydramine HCl per 30 mL, i.e. 1.67 mg/mL. For every one mL of the combination drug, we used 0.5 mL of the diphenhydramine HCl drug. So for the 1X treatment flask, 100 µL of diphenhydramine HCl was used. For the 2X flask, 200 µL of diphenhydramine HCl was used. For the 3X flask, 400 µL of the combination flask was used.

Table 3: Contents of Flasks in Diphenhydramine HCl only trial

	Control	1X	2X	3X
Diphenhydramine HCl 1.67 mg/mL conc.	0 mL of Wal-Z	0.1 mL of Wal-Z	0.2 mL of Wal-Z	0.4 mL of Wal-Z
Algae	5 mL	5 mL	5 mL	5 mL

III. Results

Six trials were conducted using the combination drug, Theraflu, that contained 21.67 mg/mL acetaminophen, 0.83 mg/mL diphenhydramine HCl, and 0.33 mg/mL phenylephrine HCl. Each trial consisted of four flasks: a control, a 200 µL dose of the drug (1X), a 400 µL dose of the drug (2X), and an 800 µL dose of the drug (3X). Each of the four flasks contained 5 mL of *Selenastrum capricornutum*. After each trial, a cell count was conducted using a hemocytometer to determine cell density and a graph was constructed.

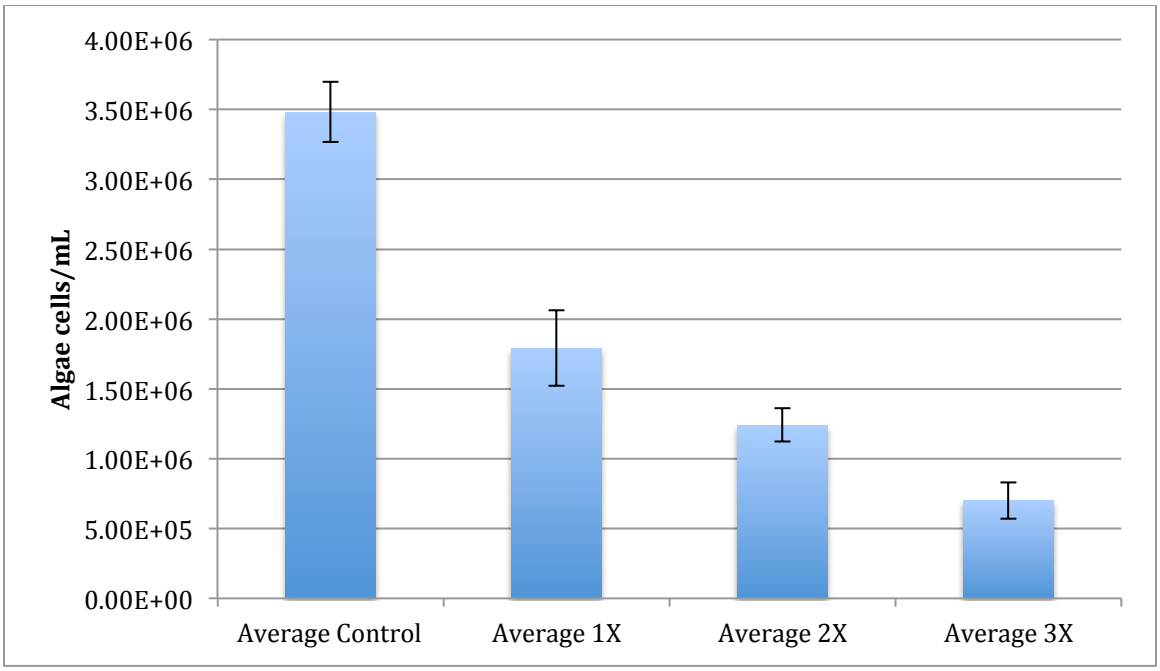
The hypothesis predicts that the graphs should illustrate a negative correlation between cell density and drug concentration, that is, as drug concentration increases, cell densities decrease. The results of the first trial, as seen in Figure 1 in Appendix A, did not completely agree with our hypothesis. As the concentration of the drug increased from 1X to 2X, the growth of the *S. capricornutum* decreased. The cell count of the 3X concentration did not agree with our hypothesis, as it had a significant increase in algal cells as compared to the 2X concentration.

However, in my lab notebook I noted that the 3X flask possibly got inoculated with 10 milliliters of *S. capricornutum* rather than 5 milliliters. This was due to the fact that the flasks were all sitting in close proximity and I lost track of which flasks I had already inoculated. The results reflected this error. Another trial was conducted.

The results of trial 2, as seen in Figure 2 in Appendix A, did agree with our hypothesis. As the concentration of drug increased, the growth of the algae decreased. This suggested that the results of trial 1 were likely due to error. We chose to run the same trial again in order to see if the same results were gathered.

The cell counts of trial 3 skewed slightly away from our hypothesis. The 1X flask contained 1.155×10^6 algal cells. The 2X flask contained 1.265×10^6 algal cells. This was inconsistent with our hypothesis that if drug concentration increased, algal growth would decrease. We chose to run the same trial again to see if the results differed.

The results of trial 4, 5, and 6 as seen in Figure 4, 5, and 6 of Appendix A, did agree with our hypothesis. As the concentration of drug in the flasks increased, the growth of the algal cells decreased, following an exponential growth pattern. Below can be seen the average cell density of each treatment in trials 2-6.



After conducting five trials that had results agreeing with our hypothesis, we decided to test the drugs acetaminophen and diphenhydramine HCl on the algae separately. Phenylephrine HCl could not be found in an over-the-counter drug by itself, so therefore it could not be tested separately.

Time only allowed for two trials of each acetaminophen and diphenhydramine HCl. In the first trial, both drugs had clumping within the flasks after seven days, including the control. Cell counts were conducted, and they also suggested that there was something within the algae that was causing the clumping.

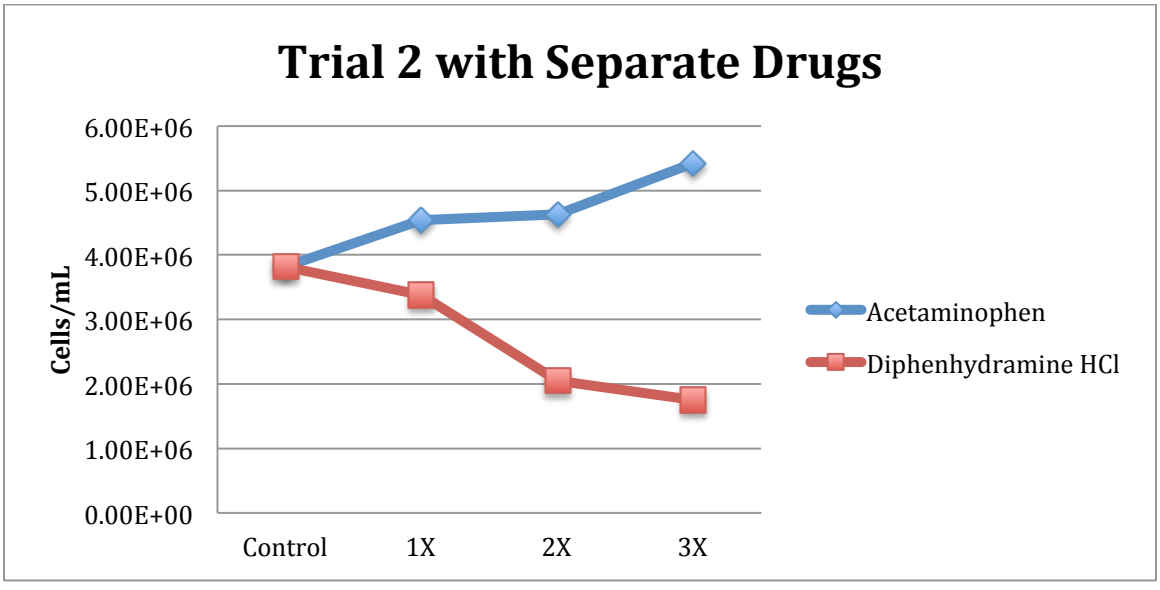
As illustrated in Figure 7 in Appendix A, there seems to be no trend within either drug trial. The acetaminophen exposed algae seemed to increase in cell count and then gradually decrease. The diphenhydramine HCl exposed algae decreased dramatically and then increased. These unexpected results are most likely due to the fact that there was unnoticed clumping in the control algae of the previous trial

that was used to inoculate the algae of this trial. This was seen visually when looking at the flask of algae. *S. capricornutum* is very sticky; it will adhere to anything foreign in the algal flasks, whether it be bacteria, undissolved drug, etc.

After learning that the control flask had clumping, we filtered the algae using membrane filtration (3). This filtered all of the suspended material and gave us clean *S. capricornutum* cultures to work with for the next trial.

A second trial using the separate drugs produced differing results. The diphenhydramine HCl trial by itself produced results that agreed with our hypothesis. As the concentration of diphenhydramine HCl increased, the growth of *S. capricornutum* decreased.

However, the acetaminophen did not yield the same result. As the acetaminophen concentration increased, so did the growth of the algae. This was most likely due to the fact that the acetaminophen was undissolved in the stock solution. When the flasks of algae were inoculated with the drug, little to no acetaminophen may have been present in the solution.



IV. Conclusions

The test results show that Theraflu—containing diphenhydramine HCl, phenylephrine HCl, and acetaminophen—inhibits the growth of *S. capricornutum* in a concentration-dependent way. As the concentration of the combination drug increased, the growth of *S. capricornutum* decreased. This indicates that there was a growth-limiting toxicant in the combination drug, commonly known as Theraflu.

When the acetaminophen and diphenhydramine HCl were tested on the algae separately, the algae clumped around the acetaminophen, preventing accurate cell counts. In the diphenhydramine HCl trial, the cell density of the algae did decrease as concentration increased. Confirming this trend requires additional trials.

The acetaminophen could have caused clumping due the fact that it was ground up from its pill form and suspended in solution before being added to the media. We had trouble getting the acetaminophen to dissolve in solution so it is very possible that the algae clumped around the small particles of undissolved drug. This may have prevented accurate suspended cell counts.

However, the diphenhydramine HCl was in liquid form. This liquid form made cells counts easier because there was no clumping. In the first trial when diphenhydramine HCl was used by itself, there seemed to be clumping present in the algae that was used to inoculate the flask. It is assumed that the clumping was caused from something in the previous week's algae culture and that the cause of the clumping was not diphenhydramine HCl. There was no clumping in the next trial after the *S. capricornutum* was filtered.

The test results strongly suggest that Theraflu—diphenyhydramine HCl, acetaminophen, or phenylephrine—may affect the environment negatively. Preliminary results show that diphenhydramine HCl alone also negatively affected the growth of the algae, suggesting that it affects the environment as well.

V. Recommendations

It is recommended that future research students study which of the three drugs in Theraflu—diphenhydramine HCl, acetaminophen, or phenylephrine HCl—is causing the inhibition of growth in the *S. capricornutum*. Future testing should use acetaminophen in liquid form, to eliminate the clumping of the algae around the undissolved drug. Also, if there is an over-the-counter form of phenylephrine HCl available, it should also be tested to see if it limits the growth of the algae. These changes will fine-tune the experiment and will better determine which of the three drugs is affecting the *S. capricornutum* negatively and thus affecting water quality and/or aquatic food webs negatively.

VI. Appendix A

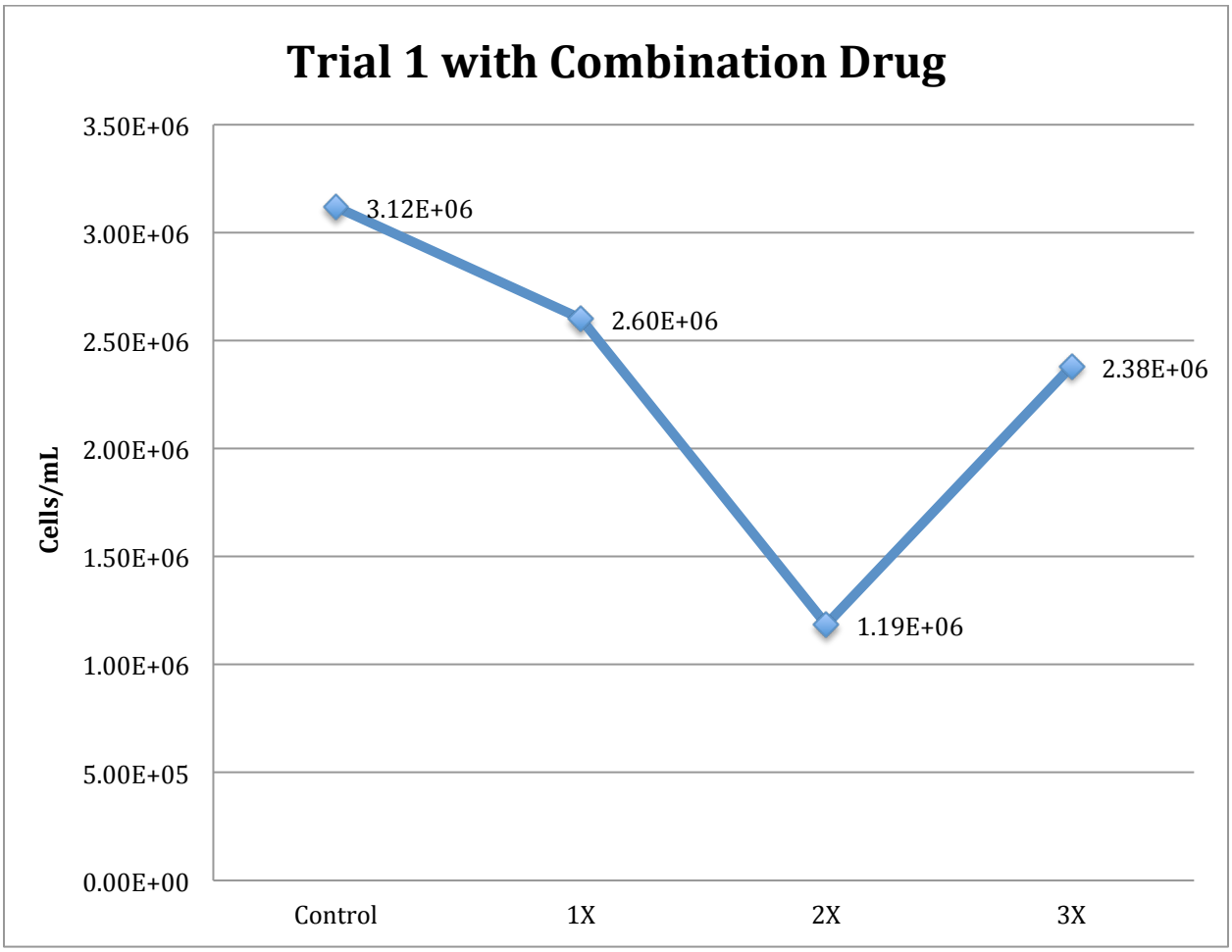


Fig 1. Trial 1 using the combination drug

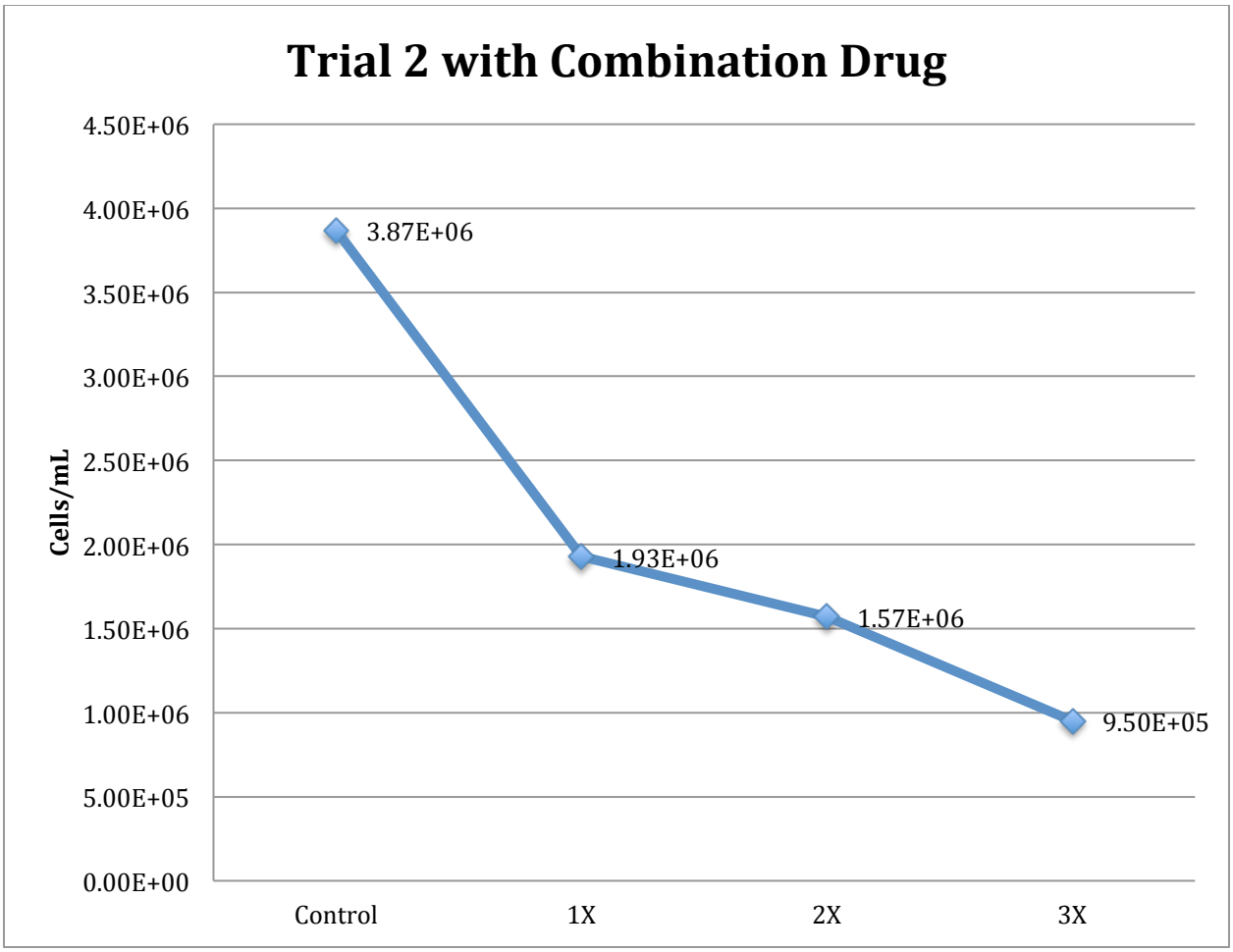


Fig 2. Trial 2 using combination drug

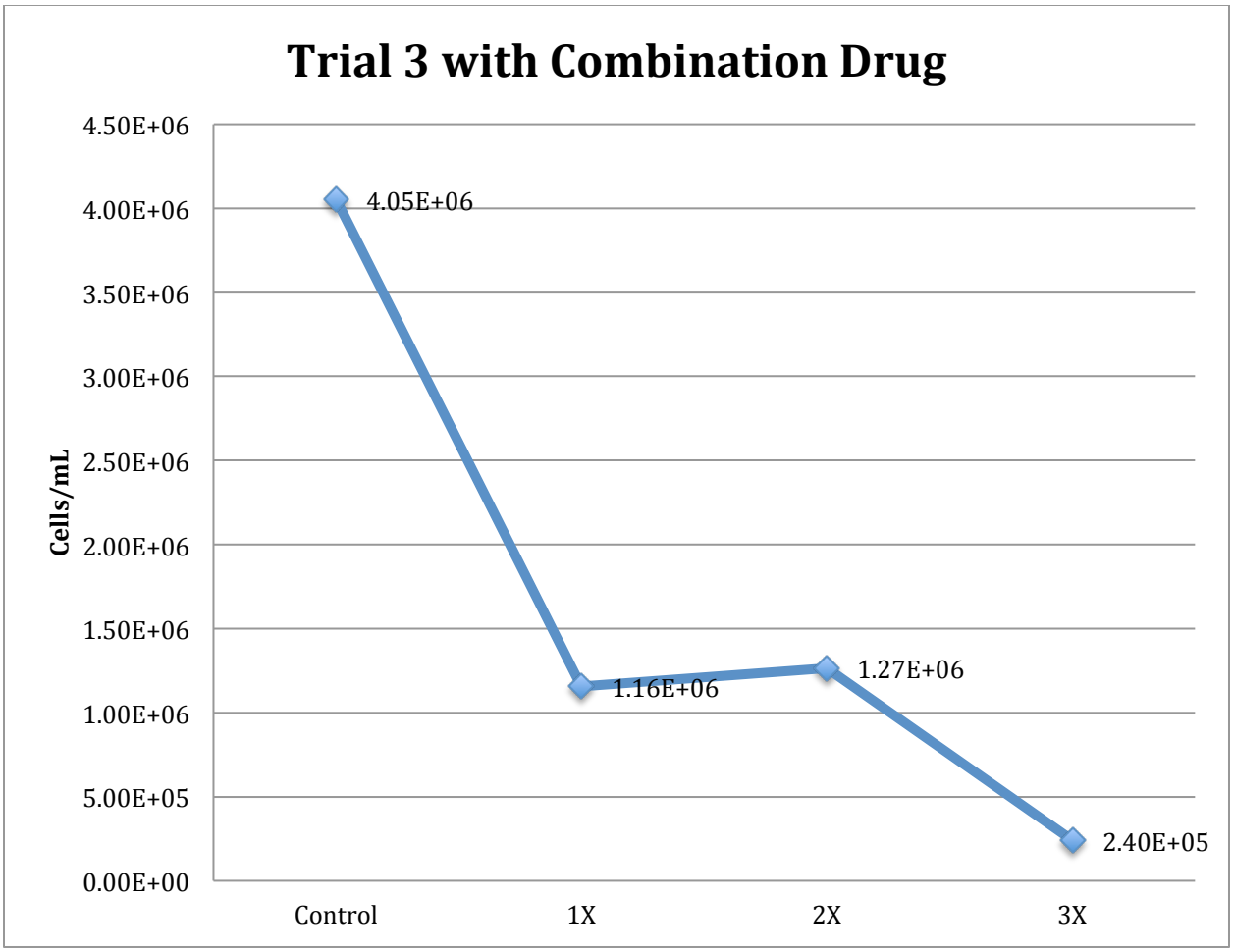


Fig 3. Trial 3 using combination drug

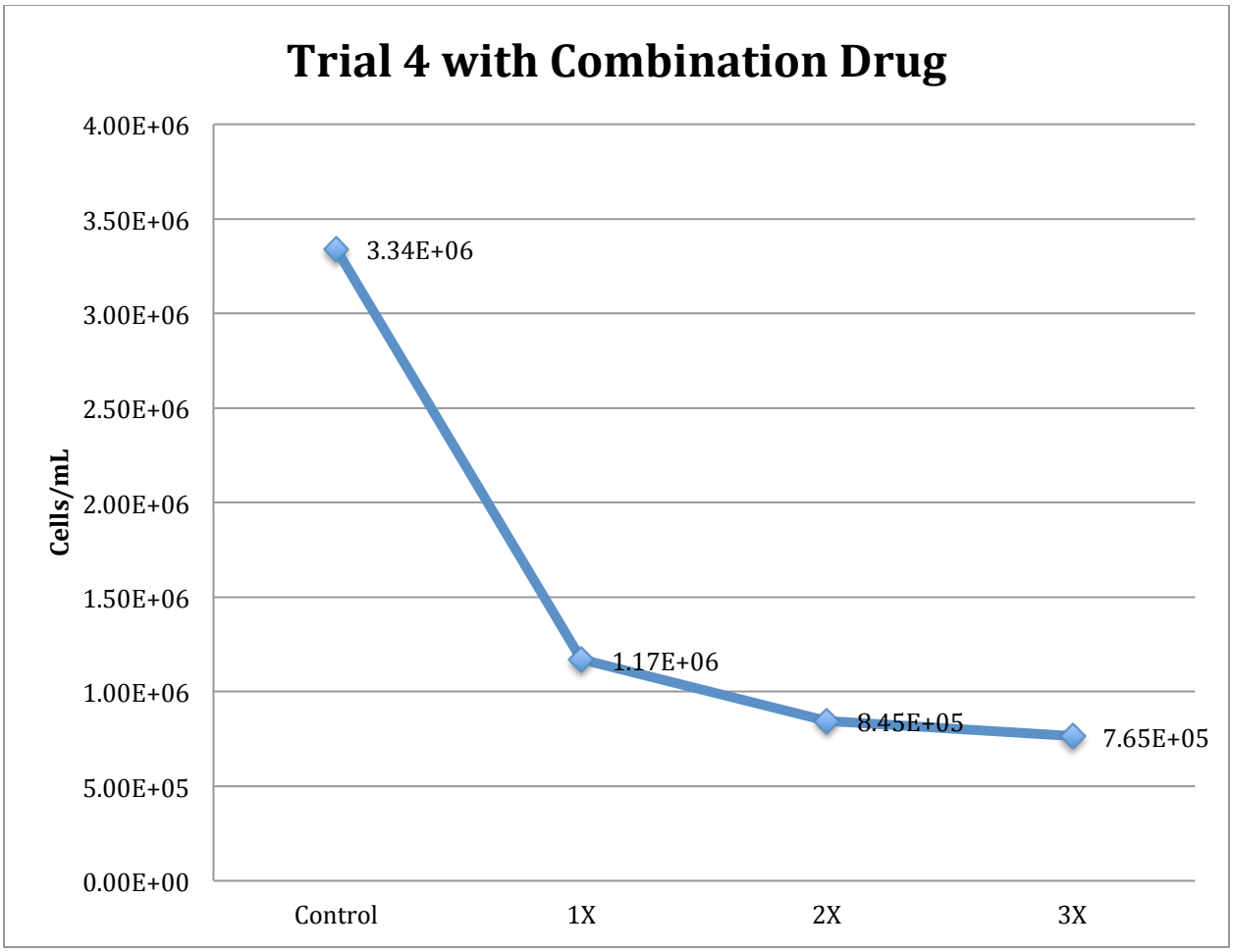


Fig 4. Trial 4 using combination drug

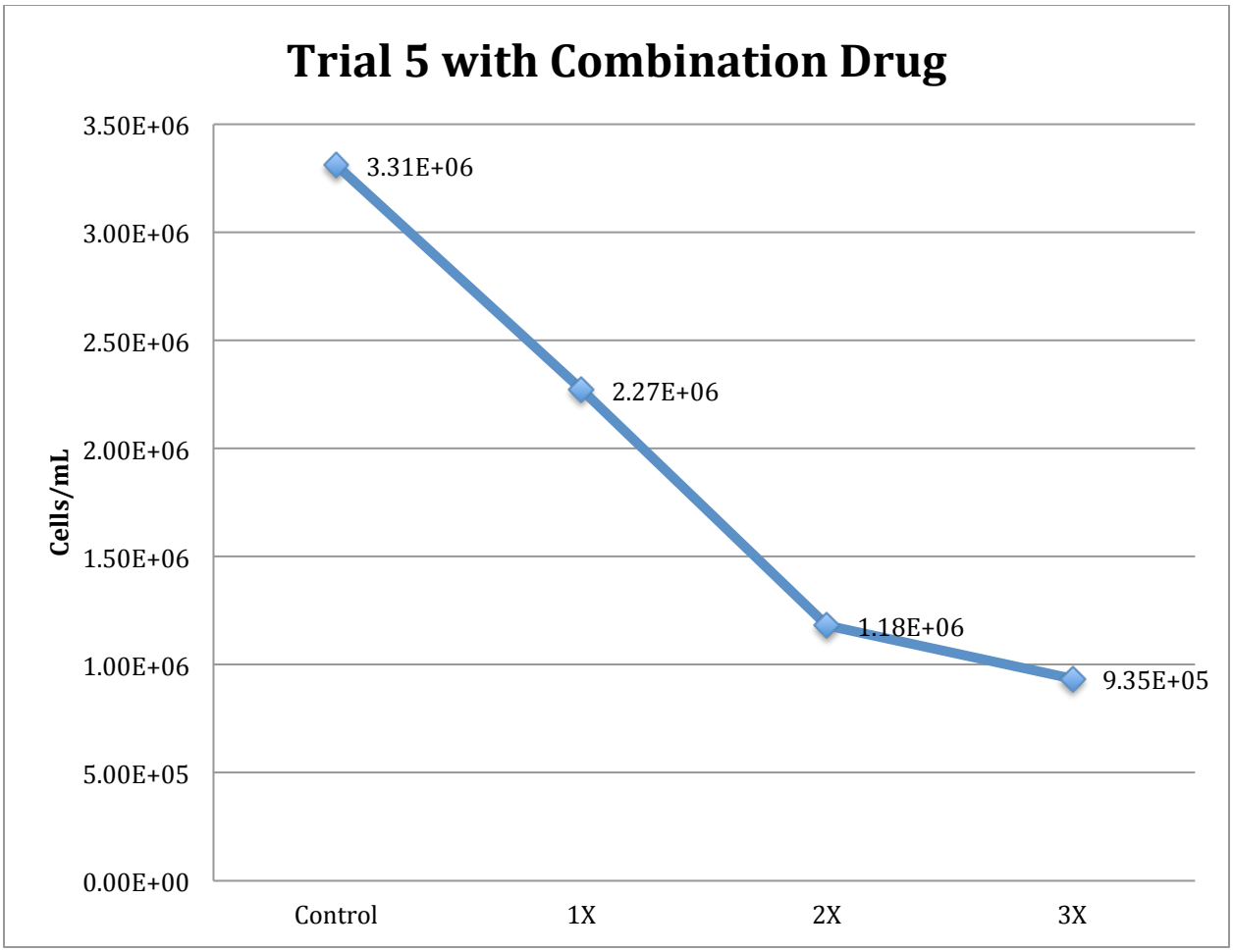


Fig 5. Trial 5 using combination drug

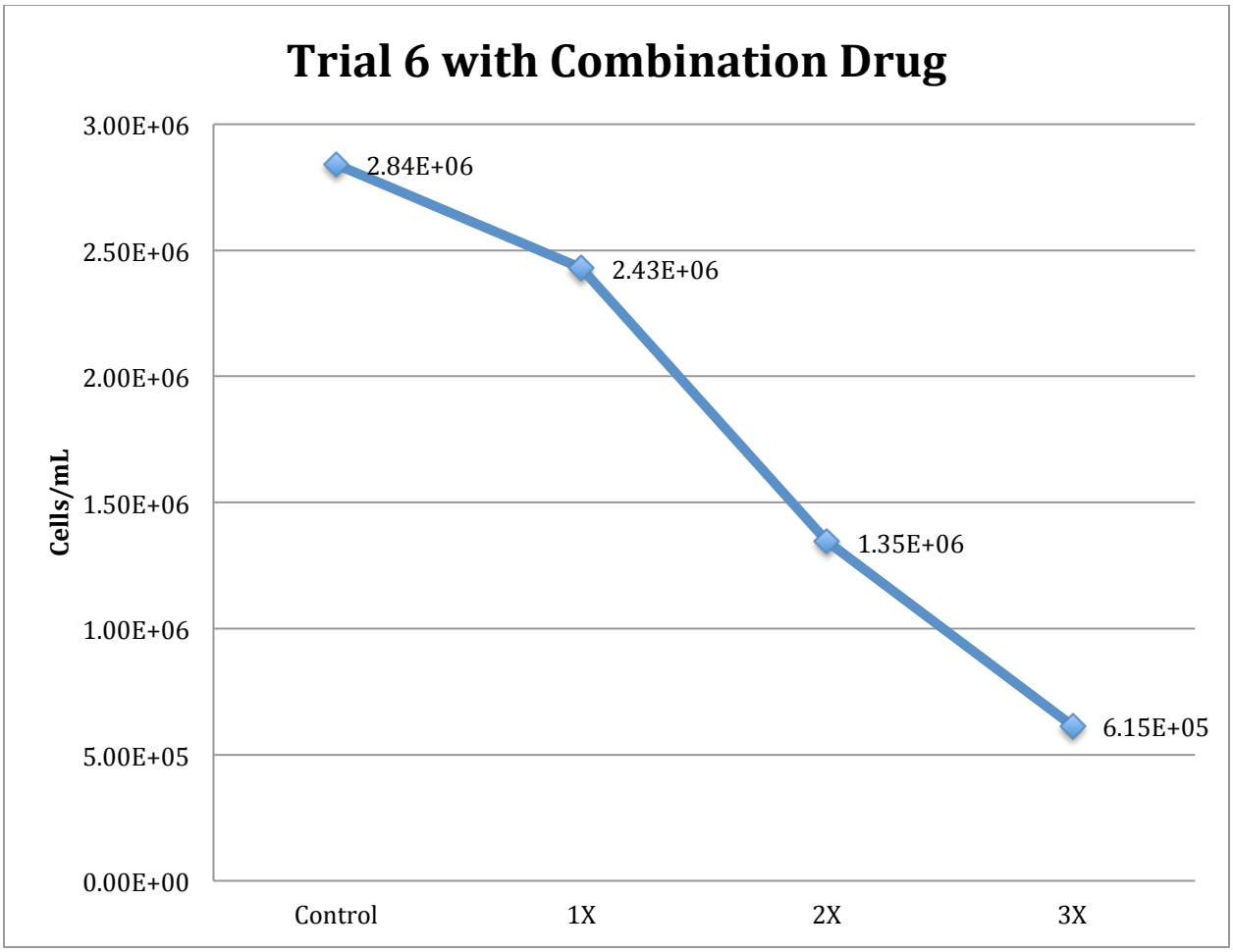


Fig 6. Trial 6 using combination drug

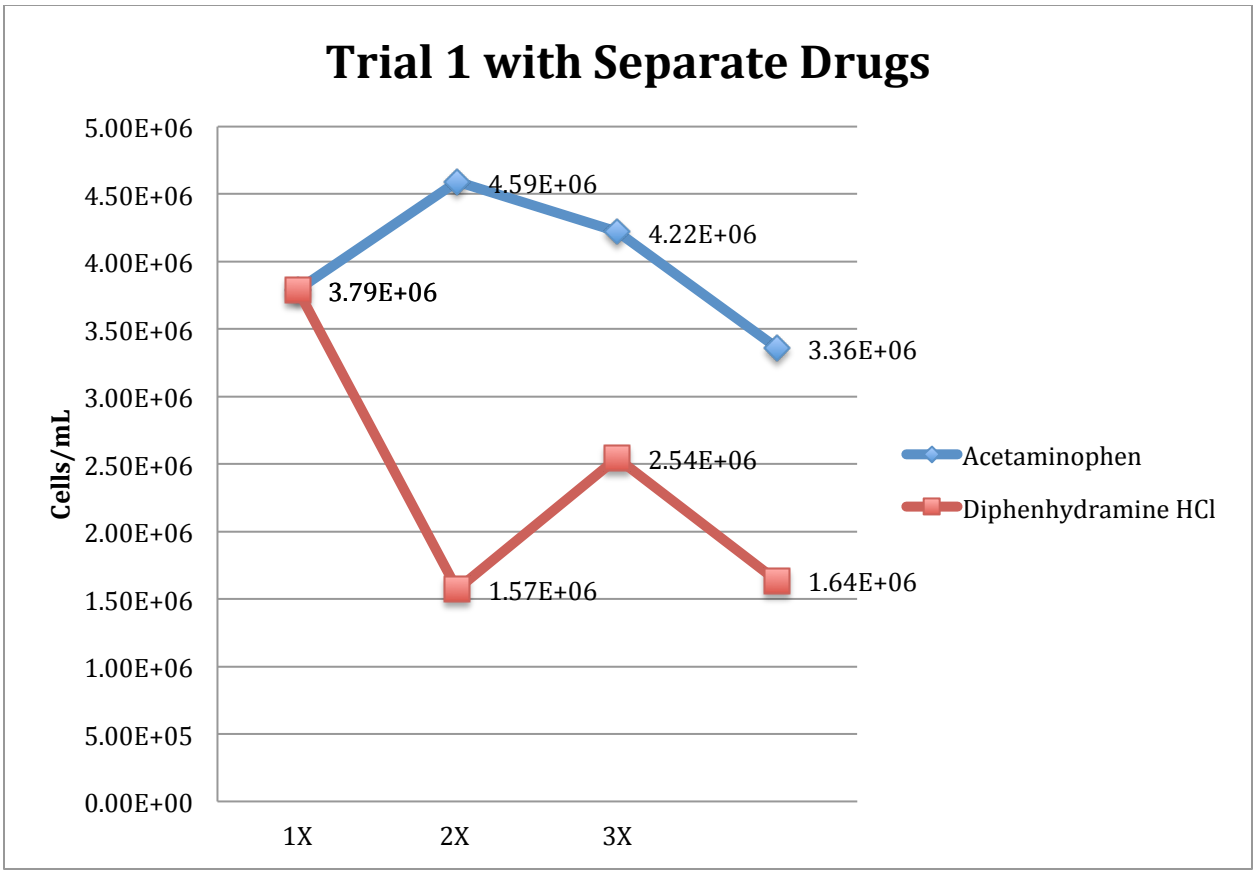


Fig 7. Trial 1 with Acetaminophen and Diphenhydramine HCl tested separately

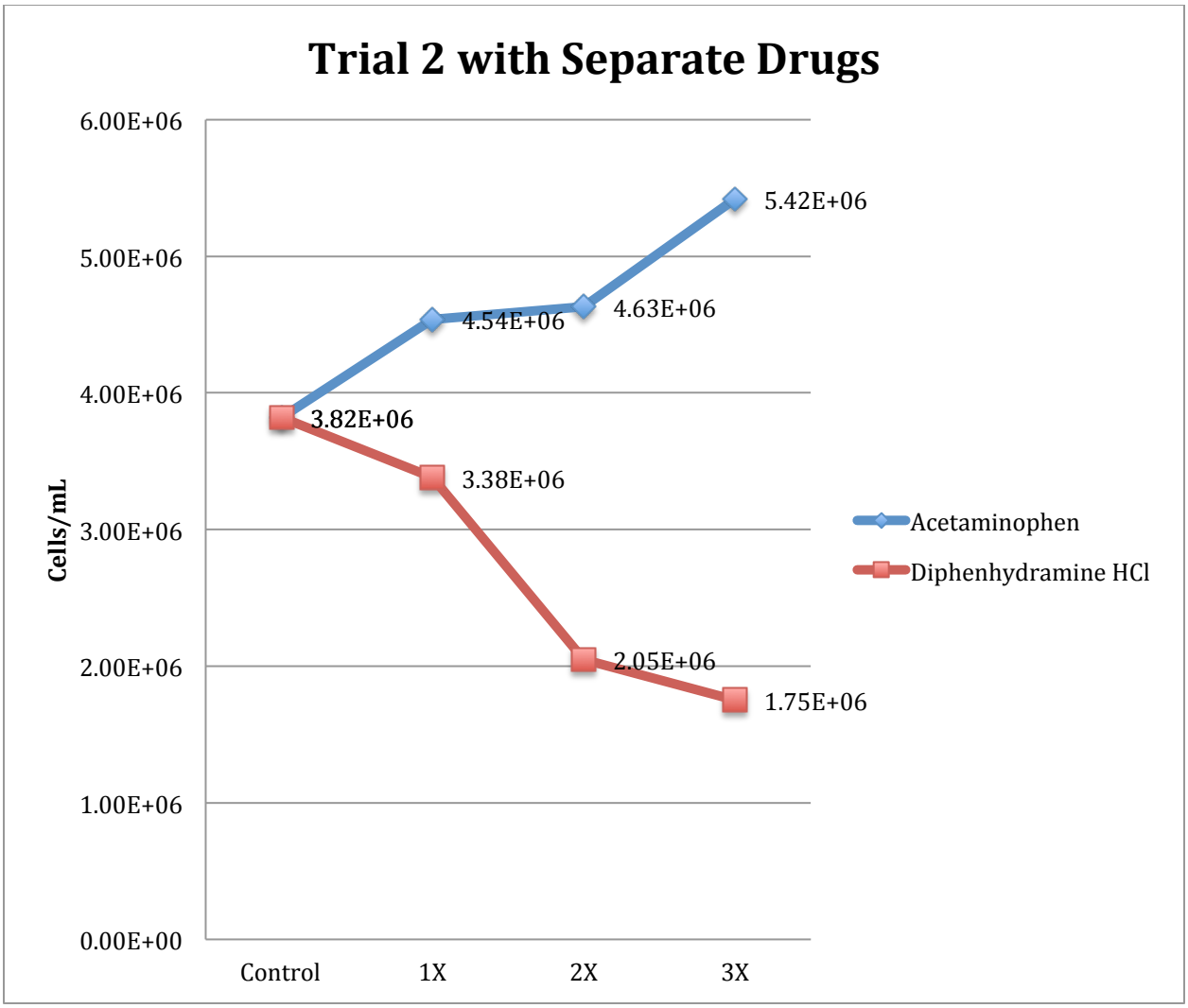


Fig 8. Trial 2 with Acetaminophen and Diphenhydramine HCl tested separately

VII. Bibliography

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