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Article

Testing the effects of resveratrol, apigenin, and glucosamine to effectively reduce prostate cancer cell proliferation, migration levels, and increase apoptosis

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SUMMARY

The current five-year survival rate of metastasized prostate cancer is only 30% and occurs in every one in nine men. Researchers have shown that people with a type of dwarfism called Laron's Syndrome are immune to cancer due to their low levels of insulin-like growth factor-1 (IGF-1). For this reason, experimentally modifying the level of IGF-1 could provide better insight into whether lowering the levels of IGF-1 in prostate cancer cell lines (e.g. PC-3) could be an effective treatment to reduce their rates of proliferation and migration and increase apoptosis. We selected three compounds, which researchers have shown decrease IGF-1 levels, to test and combine to determine which is the most promising. We conducted a cell proliferation assay in order to determine the impact of each treatment on cell proliferation. We used a migration assay to measure the migration of the PC-3 cells after each treatment. In this assay, we seeded bone marrow mesenchymal stromatolites cells (BM-MSC) underneath the PC-3 cells to induce migration. Finally, we conducted an apoptosis assay to count the number of cell deaths after treating the PC-3 cells. The compounds we used, glucosamine, apigenin, and resveratrol, can be found naturally in foods or supplements. We found the treatment with a combination of all three compounds to be most effective in decreasing cell proliferation and migration levels in the PC-3 cells. In the cell apoptosis assay, the glucosamine and apigenin combination caused the largest number of cell deaths, but an outlier in this condition resulted in a large margin of error. The combination of all three compounds resulted in the second highest number of cell deaths. With these results and further investigation, a potential new, less costly, and less painful treatment for prostate cancer could be found.

INTRODUCTION

Cancer is a fatal disease that occurs when abnormal cells in the body start to grow and divide uncontrollably, eventually spreading to the surrounding tissues. A healthy cell can turn into a cancerous cell due to a number of mutations (1). Unlike normal cells, cancer cells grow out of control and become invasive. These invasive cells continue to grow because they are less specialized than normal cells and do not have specific functions. They are insensitive to signals that normally end cell growth and apoptosis ---- programmed cell death that the body uses to remove the excess cells (2).

Prostate cancer is one type of cancer in which the cells in the prostate gland start to proliferate uncontrollably. The prostate is small walnut-shaped gland in males that produces seminal fluid that is used to make semen (3). After skin cancer, prostate cancer is the most common cancer amongst men. There were approximately 164,690 new cases of prostate cancer in the United States in 2018. Currently, once prostate cancer metastasizes or spreads, the survival rate drops to 30 percent and becomes extremely difficult to treat as surgery is no longer an option (4).

One of the factors that induces migration for prostate cancer cells is the secretome bone mesenchymal stromal cells. A study by a group of researchers at Tufts Medical Center had shown that when PC-3 prostate cancer cells are co-cultured with bone marrow mesenchymal stromal cells (BM-MSC) migrate towards these BM-MSC cells. This could be because the prostate cancer cells have the ability to promote the overproduction of bone-building cells, or osteoblasts, which then can cause osteoblastic migration (5).

Another factor that affects cancer is Insulin-like growth factor - 1 (IGF-1). IGF-1 is a hormone found in blood naturally (6). It is necessary, especially during neonatal and pubertal growth, for survival and growth of cells, suppressing apoptosis, and promoting cell cycle progression (7). The main job of IGF-1 is to regulate the effects of growth hormone (GH) (8). Upon GH stimulus, the liver works to produce this hormone. IGF-1 then binds to the insulin-like growth factor-1 receptor (IGF-1R). After binding, autophosphorylation activates the IGF-1R which proceeds to phosphorylate the insulin receptor substrate (IRS-1). The activation of the phosphate, phosphoinositide 3-kinase (PI3K) leads to the activation of Akt/PKB protein. The Akt protein then releases the anti-apoptotic protein called the Bcl-2. This P13K/Akt signaling pathway is responsible for controlling and prohibiting cell death (9).

Decreasing IGF-1 levels by a significant and unhealthy amount, growth hormone deficiency can occur. Growth hormone deficiency in adults has some clinical consequences relating to body composition and psychological well being. Growth hormone deficiency can cause an increase in abdominal fat as well as a decrease in muscle mass. These adults can also become depressed and have increased anxiety levels. Low levels of IGF-1 could also lead to many

physical deformities such as short stature. On the other hand, people with low IGF-1 levels have a non susceptibility to cancer (9).

People diagnosed with Laron syndrome have been shown to be resistant to cancer. Laron syndrome is a type of dwarfism that affects 350 people worldwide (10). This disease is a congenital autosomal recessive disorder caused by a mutation in the growth hormone receptor (GHR) gene (6). These mutations diminish hormone-receptor binding and cell signaling. Consequently, there is growth hormone insensitivity; even if the growth hormone were available, the cells would be unable to respond by generating IGF-1 which is responsible for stimulating growth and division. Insensitivity to the growth hormone prevents the growth and division that results in the development of cancerous tumors (9).

The current chemotherapeutic agents and drugs are not only extremely expensive, but painful as well. Some treatments such as radiation can cost up to $25000 while chemotherapy can cost up to $12000 a month. Many chemotherapeutic drugs are not only harmful to cancerous cells, but normal cells as well. Therefore, using chemicals naturally found in ones diet could become a valuable alternative.

In this experiment, we used three treatments, resveratrol, apigenin and glucosamine to see their effects on proliferation levels, migration, and cell death. Resveratrol is a stillbenoid made of mainly the skin of grapes and is a potential dietary compound against several cancers by regulating cell proliferation and apoptosis. A study suggested resveratrol as a potential chemotherapeutic agent when it had successfully suppressed colon cancer cell proliferation and increased apoptosis (11). In another study, resveratrol reduced the growth of prostate cancer. Resveratrol had increased apoptosis in the prostate cancer cells and inhibited wound closures, thus showing that it inhibits the invasiveness of prostate cancer cells (12).

Apigenin is a natural plant flavone found in many fruits and vegetables. It has the ability to regulate the IGF-1 to trigger growth arrest and apoptosis in prostate cancer. A 2012 study demonstrated that Apigenin is able to inhibit cellular proliferation and induce apoptosis in a variety of human cancers, including leukemia and carcinomas of the lung, skin, colon, breast, and prostate without affecting the noncancerous cells. It had the ability to inhibit P13K and Akt activation while continuing to modulate the IGF-1 signaling axis (13).

The final chemical going used in this experiment was glucosamine. Glucosamine is a naturally occurring chemical found in the fluid around the joints in the human body (14). A study showed that this chemical can inhibit the growth of human non-small lung cancer cells and negatively regulate the phosphorylation of Akt and expression of IGF-1R. Glucosamine prohibited tumor growth through reducing IGF-1R signalling and increasing ER-stress. The ER is an important calcium storage organelle; calcium influx and concentrations can effect and trigger apoptosis. Targeting the IGF-1R/Akt pathway with glucosamine could be an effective therapeutic strategy for treating some types of cancers (15).
IGF-1 is a growth hormone that once bonded, activates a series of phosphates, proteins, and processes in the PI3K/Akt signaling pathway that lead to the activation of the Akt protein which eventually result in cell proliferation. We tested three compounds, resveratrol, apigenin, and glucosamine individually and together to test their effects on prostate cancer cell lines.

We hypothesized that treatment with resveratrol, apigenin, and glucosamine would lower levels of migration and proliferation while increasing levels of apoptosis the most.
The purpose of this experiment is to determine which out of resveratrol, apigenin, and glucosamine is the leading treatment in reducing cell proliferation and increasing apoptosis in prostate cancer cells.

RESULTS

We conducted an alamarBlue apoptosis assay to compare the amount of cell deaths between the treatments and the

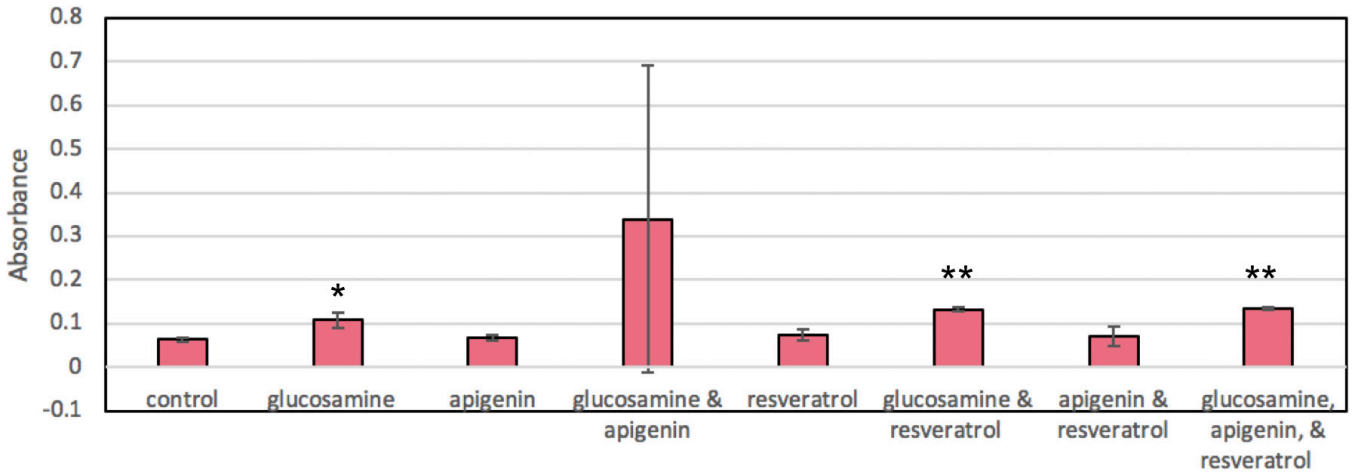

Figure 1: Comparison of cell deaths between the treated PC-3 cells after an apoptosis assay. The treatments with asterisks beside them indicate statistical significance after an ANOVA.

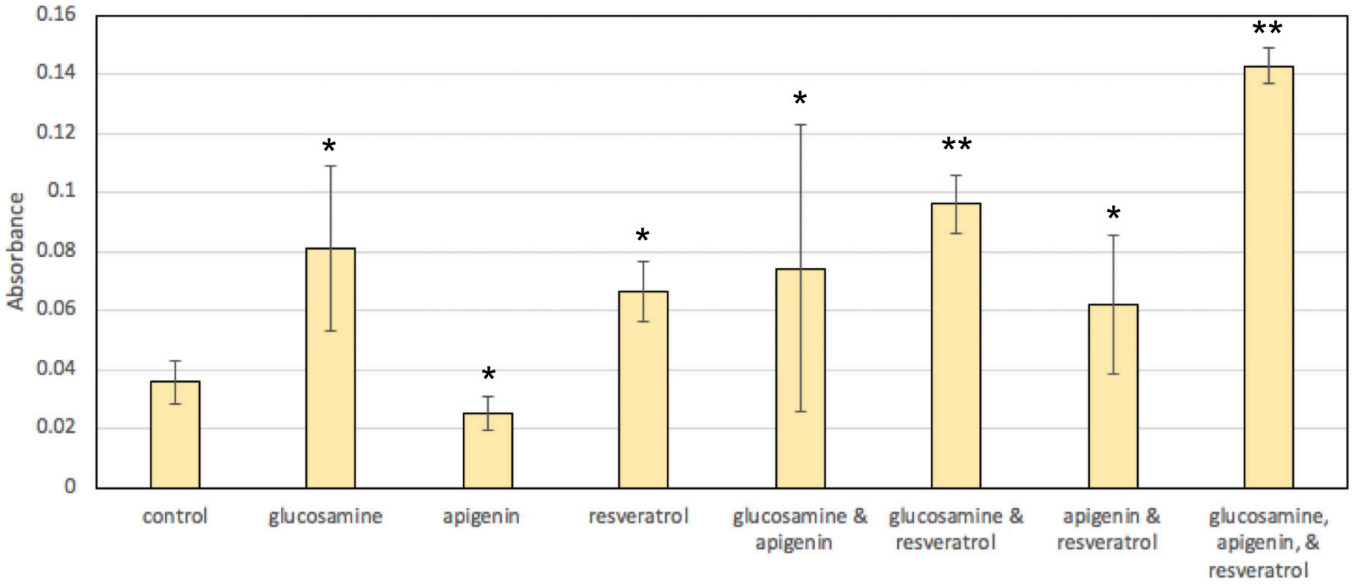

Figure 2: Reduced rate of proliferation of the treated PC-3 cells after a cell proliferation assay. The treatments with asterisks beside them indicate statistical significance after an ANOVA.

control. AlamarBlue undergoes colorimetric change as a result of a cellular metabolic reduction and measures cell viability quantitatively. We used resveratrol at a concentration at 75 micromoles, apigenin at 50 micrograms, and glucosamine at 5 millimoles. After conducting the alamarBlue apoptosis assay, as **Figure 1** had shown that the combination of glucosamine and apigenin had the most cell deaths, containing the highest absorbance amongst all the treatments. The combination group with glucosamine and apigenin contained an outlier, the alamarBlue still being vibrant compared to the other replicates where the alamarBlue solution was a lighter blue and had been more faded. Consequently, that caused an extremely large margin of error. The next most effective treatment had been the combination of all three chemicals: the glucosamine, apigenin, and resveratrol combination, which had a smaller margin of error and is more reliable. Amongst the treatments, the only treatments that showed significant values were glucosamine, glucosamine and resveratrol, and the combination of all three treatments. Glucosamine had a p-value of 0.0209, apigenin only had a p-value of 0.2332, resveratrol only had a p-value of 0.1262, glucosamine and apigenin had a p-value of 0.1532, resveratrol and glucosamine had a p-value of 6.8376E-05, resveratrol and apigenin had a p-value of 0.3075, and the combination of all the treatments had a p-value of 3.8211E-05.

We conducted a proliferation assay to compare the slowed down rates of proliferation with the negative control. The results from the MTT cell proliferation assay had shown that the glucosamine, apigenin, and resveratrol combination was most capable in reducing PC-3 prostate cancer cell proliferation, with a small margin of error as shown in **Figure 2**. All the treatments were significant with glucosamine with a p-value of approximately 0.0485, apigenin with a p-value of approximately 0.0250, resveratrol with a p-value of 0.0493, apigenin and glucosamine with a p-value of 0.0037, resveratrol and glucosamine with a p-value of 0.0007, resveratrol and apigenin with a p-value of 0.0422, and the combination of all three treatments with a p-value of 2.5022E-05.

We conducted a boyden chamber migration assay in order to exhibit the rates of migration before and after treatment. Finally, after the migration assay, the treatment of the combination of all three chemicals had the least number of cells left in the membrane of the inserts exhibiting the fact that it was most successful in being able to reduce the migration of the PC-3 prostate cancer cells as seen in **Figure 3**. In addition, all the ANOVA tests conducted had yielded statistically significant p-values. Glucosamine had shown a p-value of 0.0006, apigenin had shown a p-value of 0.0212, resveratrol had shown a p-value of 0.0003, apigenin and glucosamine had shown a p-value of 0.0005, resveratrol and glucosamine had shown a p-value of 4.1166E-05, resveratrol and apigenin had shown a p-value of 0.0005, the combination of all three treatments had shown a p-value of 0.0002.

DISCUSSION

All the chemicals tested were able to successfully induce apoptosis and inhibit proliferation and migration. However, they were most effective when used in combination, concluding that the combination of these chemicals could lead to a novel, cost effective treatment for prostate cancer in men.

To further investigate, we could test these treatments on other cancer cell lines. Breast cancer is another type of cancer closely linked with IGF-1; therefore, these medications could become a potential treatment. Many cancers such as small intestine cancer, soft tissue cancer, bladder cancer appear following prostate cancer. In addition to prostate cancers, we could test these treatments on second cancers.

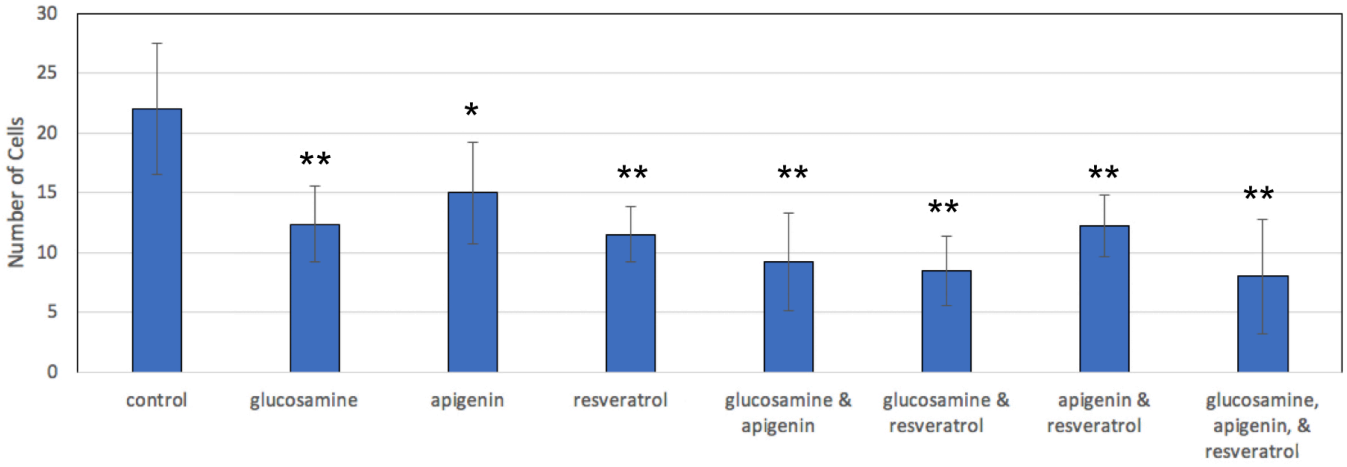

Figure 2: Reduced rate of proliferation of the treated PC-3 cells after a cell proliferation assay. The treatments with asterisks beside them indicate statistical significance after an ANOVA.

With further research, these results could lead to a more efficient and less costly possible treatment than existing treatments for prostate cancer patients.

Additionally, these treatments could treat prostate cancer in animals as well. Prostate cancer appears and develops in many dogs over eight years old. Fifty percent of dogs develop cancer at some point in their lifetime which cannot be treated with cancer. The most effective treatment currently is radiation combined with chemotherapy which only gives them an extension survival time of twenty months. Therefore, these treatments could potentially treat these animals.

We could conduct further research by beginning to use animals as model organisms. Besides humans, dogs are the one of only other species that prostate cancer has significant incidence in. Specifically, the Bernese mountain dog, Irish wolfhound, and Saint Bernard are the breeds most susceptible to fatal tumors. To continue this experiment, each treatment could treat dogs with prostate cancer to test whether the results found in this experiment replicate in a living organism.

Another potential animal test model for further research is immunodeficient mice. These mice can be genetically modified to mimic and develop this human disease. We could give potential treatments to these mice to test which treatment would be most effective in living organisms. Further in vivo testing is essential to see if the cancer cells react the same way they did in vitro before using these drugs on people.

There were multiple limitations that were present in this experiment. In order to minimize human error and show more significant results, we would conduct more treatments. Due to the limited time and budget, this was not possible. Both the PC-3 prostate cancer cells and the HS-5 bone marrow mesenchymal stromal cells (BM-MSC) were susceptible to contamination because of yeast and bacteria. As a result of restricted time we could not conduct more replicates of the assay because of the limited number of cells that could be grown within the set period of time. Another limitation that occurred as a consequence of few numbers of cells, we could not use more cells per well in each assay.

There were various shortcomings during this experiment. When creating the treatments, miscalculations could have occurred in determining how many milliliters we needed to make each treatment when converting from moles. Additionally, we used pipettes, which are not the most accurate or precise form of measurement. A small margin of error could have also occurred when micropipetting the solutions into the wells for experimentation. Moreover, the cell counts can only be an approximation rather than an exact number; each well would have slightly different numbers of cells to start with.

In essence, the results from this study could aid in discovering a treatment for other types of cancer. Specifically, cancers that develop following the prostate cancer such as small intestine cancer, soft tissue cancer, bladder cancer and other second cancers. Other potential cancers that we could treat with the results from this investigation are cancer related to IGF-1 such as colon, pancreas or breast cancers.

MATERIALS AND METHODS

The medium for cell culture was made using RPMI 1640 with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/ Streptomycin (P/S). The treatments were made using dimethyl sulfoxide (DMSO), the medium previously made, resveratrol, apigenin, and glucosamine.

For the cell proliferation assay, we seeded PC-3 cells into a 96-well plate at a density of 5 x 10^2-10^5 cells/well in 10 µl of culture medium with and without compounds. The cells were culture in a CO2 incubator at 37 C for 24-48 hours. We added 10 µl of the MTT reagent to each well using a repeating pipettor. We then mixed the cells gently for one minute on an orbital shaker. We incubated the cells for 3-4 hours at 37 C in a CO2 incubator. After incubation, the formazan produced in the cells appeared as dark crystals in the bottom of the wells. We added 100 µl of crystal dissolving solution to each well, and incubated for 4-18 hours in a 37 C CO2 incubator.

This solution dissolved the formazan crystals and produced a purple solution. We measured the absorbance of each sample at 570 nm using a microplate reader.

Following the cell proliferation assay was an alamarBlue apoptosis assay. We seeded the cells at 5,000 per well in a 96-well plate in 100 µL of medium. We incubated the cells in a 37 C incubator for 24-72 hours. We added 10 µL of the alamarBlue reagent directly to cells. We incubated the cells for 1-4 hours at 37 C incubator and protected from direct light. We monitored the absorbance of alamarBlue at 530 nm.

The last assay conducted was the migration assay. First a homogenous cell suspension of the bone mesenchymal stromal cells was made. Then we seeded 10,000 - 50,000 stromal cells per 24 well in full growth medium. Then a homogenous cell suspension of PC-3 cancer cells was made. We seeded at 5000 - 20,000 cancer cells per 24 well plate in semipermeable trans-well inserts in full growth medium above stromal cells. The 24-well plate for migration assay was set up with the lower chamber containing the stromal cells and the top chamber containing a 200 µl suspension of PC-3 cells in full medium for 24-48 hours to allow for migration. To assess the number of migrated cells, we transferred the membranes to a new 24-well plate and fixed for 10 minutes in a 10% formalin solution. We stained the cells with crystal violet dye to resolve cells on membranes. We submerged them for 5-10 minutes in the staining solution before we washed the membranes in a beaker of water. We dried the membranes before we mounted them onto microscope slides. We quantified the migration by counting 5 representative fields at 10x magnification.

To analyze the data and determine its statistical significance, we conducted ANOVA and Tukey post hoc tests to test the significance.

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Article

A Scientific Investigation of Alternative Growing Methods to Cultivate *Lactuca sativa*

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SUMMARY

Hydroponics is the process of planting seeds inside a solid growth medium instead of soil and providing them with nutrients that soil would normally provide. Aquaponics is a method of growing plants without soil, utilizing fish waste to fertilize plants. In this experiment, we compared both methods to observe if hydroponic nutrients and fish waste would produce plants with different heights when growing a widely cultivated vegetable like *Lactuca sativa*. Our hypothesis was that aquaponics would be a more efficient farming method in terms of growth per day and average height, since the method uses a natural fertilizer. The aquaponics system would simulate an open environment, helping the plants better adapt to the natural fertilizer provided by fish. At the beginning of the experiment, the plants in the aquaponics system were taller than those in the hydroponics system, but the hydroponics plants had a faster growth rate than aquaponics plants by the end of the experiment. However, the aquaponics system had a higher growth rate than the hydroponics system in the majority of the experimental timeframe, and had a higher average plant height. Therefore, the aquaponics system was a superior system to the hydroponics system, producing plants with better height by 0.4 centimeters on the final day, and higher average growth rate by 0.02329545455.

INTRODUCTION

Aquaponics and hydroponics are similar methods of growing plants, though it is unknown which method is superior in cultivating edible plants such as lettuce. Aquaponics and hydroponics are still not as heavily used as traditional farming methods like organic farming, multiple cropping, and other farming methods that rely more heavily on soil and land (1). Because of their unpopularity, not much is known about these alternative growing systems, and a majority of farmers do not know the advantages that aquaponics and hydroponics have to offer, including a lower water requirement, a shortened growing period, and indoor compatibility (1, 2). Despite the high cost of required resources to produce a large-scale aquaponic or hydroponic system, both systems are appealing alternatives for farmers with less access to material resources (1).

Aquaponics and hydroponics need 90% less water than regular soil systems to operate (2), and allow plants to potentially grow faster than the regular household soil-plant (1, 2). The plants and fish in an aquaponics system have a symbiotic relationship in which the fish provide droppings, which the plant filters from the top of the aquarium to absorb their nutrients (1). The fish are protected from ammonium and nitrite spikes by the plants converting nitrogenous waste to nitrates (1). Hydroponics systems have a similar arrangement to an aquaponics system, though the nutrients are supplied to the system by pouring hydroponic nutrients in the water instead of having fish in the reservoir (1). Problems from growing plants using soil outdoors such as temperature, insects, weeds, overwatering, and high physical labor are remedied through using hydroponic or aquaponic systems (1).

There are currently three prominent ways to grow plants using hydroponics and aquaponics (3). One of them is media-filled beds, a system that utilizes a grow bed and a reservoir, with a pump bringing the nutrient-filled water to the plants in the growth bed (3). Another is the nutrient film technique, a system with plant roots hanging down through holes in a PVC pipe. A reservoir holds the nutrient-filled water with hydroponic nutrients or aquaponic waste from fish, which is pumped through the pipe. The pumped nutrients allows the plant roots to absorb all of the nutrients directly from the flow of water (3). The last system is deep water culture, a system that relies on having a styrofoam raft on top of a reservoir and plant roots hanging down from the raft into the reservoir to easily absorb nutrients. This system is also known as the raft system and is predominantly used for growing small plants (4).This experiment was suited for the raft system, as it is specialized in growing small plants like lettuce. Based on the experiences of teachers and students at Terra Nova School of Science and Sustainability, goldfish would accompany the aquaponics system because they produce a higher amount of waste than the average fish.

We hypothesized that the aquaponics system would be superior to the hydroponics system because the aquaponics system contains fish, producing a natural source of fertilizer for the plant. We propose that the lettuce will prefer a natural fertilizer like fish waste instead of an artificial one made by humans, which the plant would have to adapt to in order to grow since it's not a natural organic process. The cells inside the lettuce plant will be able to use the natural fertilizer with ease, meaning that cell replication will be faster when the plants are in an environment that simulates those of a wild lettuce plant

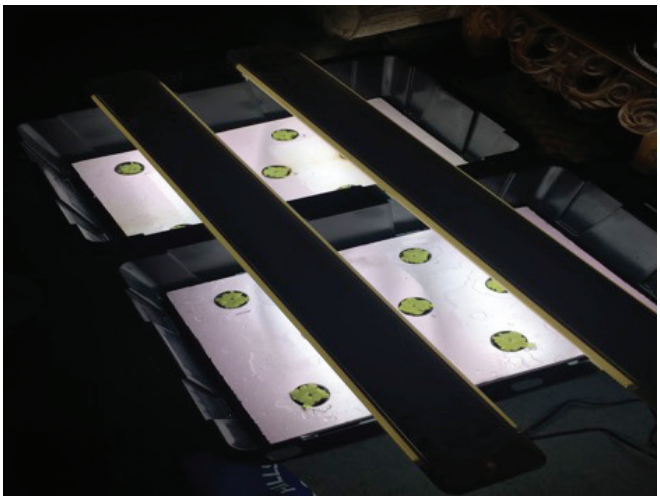

Figure 1: Picture of experimental setup on the first day. *L. sativa* seeds planted inside rockwool within a Styrofoam raft. Net pots contained LECA stones inside of raft systems with LED lights shining on both systems

near a body of water populated by fish. Existing literature provides comparisons between hydroponics or aquaponics to traditional farming methods that utilize nutrients from the soil, but not with each other. Therefore, this experiment was conducted in order to increase knowledge of comparisons of aquaponics and hydroponics performance.

RESULTS

This experiment was conducted to compare hydroponics to aquaponics. The data was collected through measuring the *L. sativa* plants inside both systems every day. Both systems had a black, plastic, reservoir holding water with a Styrofoam raft carrying the plants inside, along with LED lights to ensure the plants could photosynthesize (**Figure 1**). The Styrofoam raft contained small holes where net pots were put in which contained rockwool and Lightweight Expanded Clay Aggregate (LECA) stones as a medium for the *L. sativa* plants. The net pots contained small holes to expose the roots of the plants for them to absorb the nutrients in the reservoir. Water was then squirted in small amounts on each seed to initiate germination. Water was added to the reservoirs every week so that the water in each reservoir didn't completely evaporate. Comparing side by side observations and measurements showed which system (hydroponic or aquaponic) would produce a higher yield of a small plant such as *L. sativa*.

With the height data gathered over two weeks, the mean and growth rate were calculated for all the plants inside both systems for each day the experiment was conducted. The mean showed that the aquaponics system had a higher average plant height by 0.4 centimeters after two weeks (**Figure 5**). The aquaponics system had a slightly higher average growth rate of 0.2528409091, while the hydroponics system had an average growth rate of 0.2295454545 by the end of the experiment (**Figure 6**). The aquaponics system

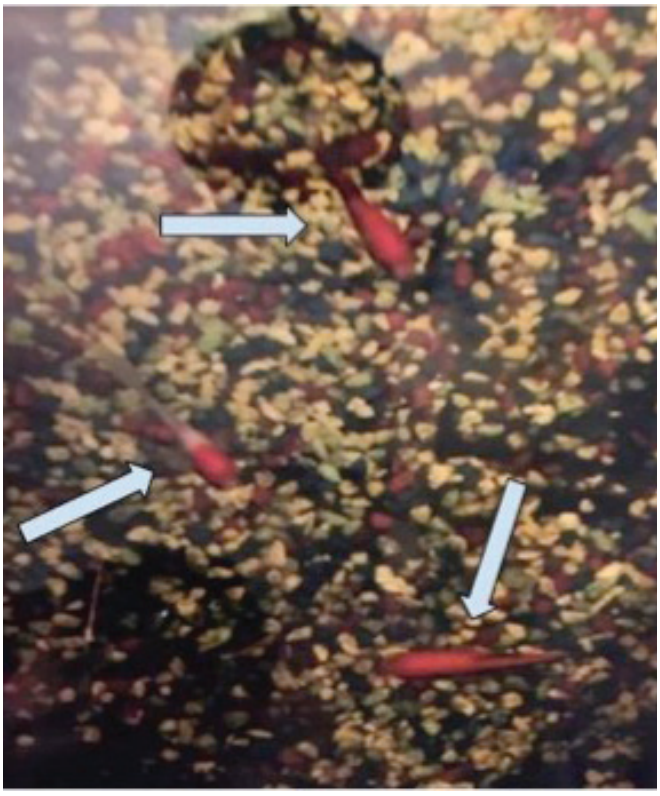

Figure 2: Picture of the inside of the aquaponics system. The system contained three Carassius auratus with brightly colored pebbles on the floor of the reservoir.

also had a standard deviation of 1.283035635, while the hydroponics system had a standard deviation of 1.20896502

The aquaponics system had a higher average plant height, and a higher average growth rate during days 3 through 13 (**Figure 5**, **Figure 6**). The aquaponics system yielded higher plant height averages (**Figure 5**), but the hydroponics system had a higher growth average on day 14 (**Figure 6**), the same day that the hydroponic nutrients were

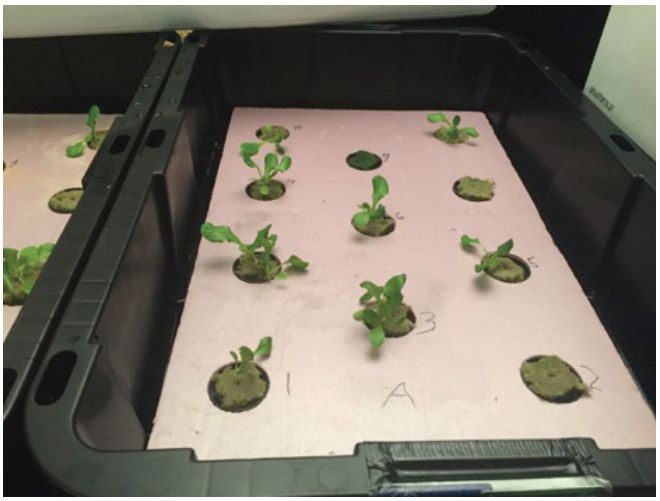

Figure 3: Picture of the aquaponics system on the eighth day with a Styrofoam raft and 11 plants inside contained in rockwool. Plants 2, 8, and 9 contained defective seeds. LED lights were removed for better photography and easier conducting of plant measurements.

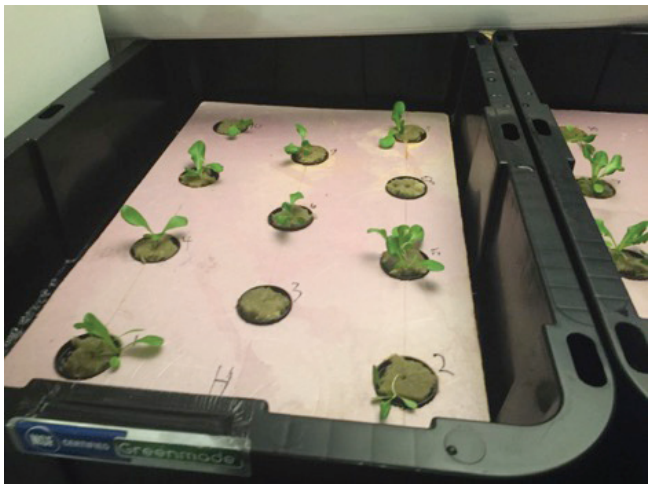

Figure 4: Picture of the hydroponics system on the eighth day. LED lights were momentarily turned off to enable better photography and easier plant measurements. Plants 3 and 8 contained defective seeds.

added to the hydroponics system before measuring plant heights.

DISCUSSION

Given our results in which the average plant height in the aquaponics system was higher than in the hydroponics system, our experiment supported our hypothesis. We hypothesized that the lettuce plants in the aquaponics system would grow quicker due to the aquaponics simulating a natural environment and because the hydroponics system had nutrients the lettuce plants might have to adapt to. The data supported the claim that the hydroponics plants adapted to the hydroponics nutrients slower than the aquaponics system, and then treated the nutrients the same way an aquaponics plant would treat fish waste after adapting. The hydroponics plants have better growth rates once they have adapted to their environment, unlike aquaponics plants which adapt immediately (**Figure 6**). We believe it's because of how their environment is simulated to be the same as a wild, natural lettuce plant near a body of water populated with fish.

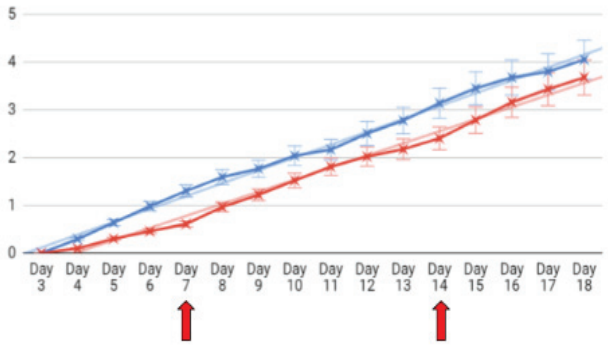

Figure 5: Graph of the average height from the 11 plants in each aquaponics and hydroponics system over time with trendline. R-squared values represent the distance from the trendline to the original graph. Error bars represent standard deviation of the plant heights.

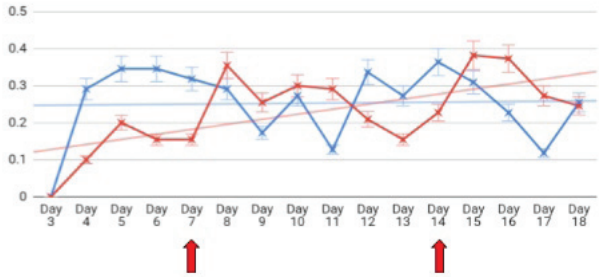

Figure 6: Graph of the average growth rate in centimeters of 11 plants in each aquaponics and hydroponics system on each day when conducting the experiment, along with error bars and a trendline with the R-squared value

While the experiment ended early in the lettuce plant's life cycle, that data can be used to determine their future height. If the plants continued to follow their pattern in height until their harvest, our conclusion of aquaponics as a better growth method wouldn't change.

The aquaponics system is superior to the hydroponics system, as the aquaponics system has a sustained higher growth rate through day 1 and day 13 (**Figure 6**) and had a higher plant height average (**Figure 5**). Therefore, the aquaponics system was better than the hydroponics system in plant height and growth, determining that the superior method of fertilization for plants is aquaponics.

Although our results suggest that the aquaponics system is the faster method for growing lettuce, there are still a few factors we have to take into consideration. For example, goldfish produce more waste than other fish. Using the same system with an alternative fish that is compatible with human consumption but produces less waste might change the results. If the experiment were longer, it would be possible that the hydroponics system could surpass the aquaponics system in height, using a trendline to predict the future average growth rate.

Not everything in the procedures went as smoothly as possible, and some human error may have affected the plant growth in both the hydroponics and aquaponics systems. For example, the amount of nutrients given to the hydroponics system could vary. The plants may have had too few nutrients for the hydroponics system to grow to expected sizes because the reservoir was larger than the one used in a previous experiment. We gave the same amount of nutrients that were used previously on the first week (one teaspoon), meaning the fertilizer was diluted in a larger volume of water. Another data fault was the living quarters of the three goldfish. Because of the use of a raft system, the plant roots were exposed into the reservoir. The roots tempted the fish to nibble at them, stunting the plant growth of the aquaponics system, and could have caused the growth rate of the aquaponics system to be lower than the hydroponics system at day 14 (**Figure 6**).

Aquaponics and hydroponics are systems that can grow plants of varying sizes and testing each plant out to see which system it is suited for is important. Using this

experimental template, future research on these systems of plant and fish could include: growing different plants, and most importantly, seeing the types of phenomena that can emerge. For example, of the three main systems described in the introduction for aquaponics and hydroponics, we only studied one type of system, one type of fish, and one type of plant in this experiment. There are hundreds of combinations to experiment and discover what systems would be most optimal for all circumstances, and all combinations can be turned into further experimentation.

MATERIALS AND METHODS

Design and construction of a reusable fuel cell frame

A goldfish requires at least 19 liters of water to survive, so to make the system successful, a reservoir that could carry 75 liters of water would be required. Holes were drilled into a Styrofoam raft that was placed on the surface of the water inside the reservoir. Net pots were placed inside the holes. Lightweight Expanded Clay Aggregate (LECA) stones and rockwool were put inside the net pots of the reservoir which contained seeds. The previous steps were repeated to build the hydroponics system using an identical reservoir, Styrofoam raft with drilled holes, and net pots with solid substrate. Eleven seeds were planted in each styrofoam raft inside both systems, then a drop of water was squirted on each seed. Three goldfish were placed inside one of the filled reservoirs to create the aquaponics system. A teaspoon of hydroponic nutrients were added to the hydroponics system every seven days. Two LED lights were set above and shared between the two reservoirs to ensure that both systems had an equal amount of light. The amount of water added to both reservoirs each week was 3.8 liters. Every day, the three goldfish residing in the reservoir were fed as much as they could eat for two to three minutes using standard fish flakes that contained the proper nutrients for goldfish.

The height of the *L. sativa* plants were measured with a ruler in centimeters, and the data were logged using a data management tool that calculated the average mean and growth rate of all plants in both systems. Any of the plants that did not grow in the experiment were given a value of zero in all data and graphs.

ACKNOWLEDGMENTS

When going through this scientific investigation of finding an alternative sustainable food source, some special persons helped contribute. I would like to thank my peers in my science class for supporting my goals and helping edit my article to meet professional standards; my teacher, for assisting me by providing ample time, research, encouragement, and experts on the subject to help aid my investigation; the group of experts, including the wonderful students and teachers at Terra Nova School of Science and Sustainability, who helped me tremendously by providing me with information that I could use for both aquaponics and hydroponics. Finally, I would like to thank my parents, for providing all of the different materials that I couldn't get my hands on, and giving me all of the love and support that I could ever need when attempting to reach my goals.

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Article

Analysis of monotherapy and combination therapy on *Helicobacter felis*

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SUMMARY

Helicobacter felis **is a bacterium that infects the gastrointestinal systems of small animals (most commonly cats), causing stomach gastritis with symptoms of vomiting, dehydration, poor appetite, pain and weakness. The goal of this study was to determine which antibiotics would be most effective at eradicating *H. felis*, therefore promoting healing in afflicted animals. It was hypothesized that, in a 24-hour period, a combination of high-performing antibiotics would be more effective in combating infection than monotherapy with a high-performing antibiotic. In the current study, we gathered various antibiotics that have been proven to successfully fight bacterial infections, including ampicillin, gentamicin, streptomycin, tetracycline, chloramphenicol, and oxacillin. We also created a combination of gentamicin and streptomycin, the two most effective monotherapy antibiotics, in addition to a combination of ampicillin and tetracycline, the two most ineffective monotherapy antibiotics. The *H. felis* was grown on tryptic soy and subjected to the Kirby-Bauer antibiotic test with eight different antibiotic treatments. After 24 hours of incubation, we measured the inhibition zones of the stand-alone monotherapy antibiotics (i.e., how effective they were against the *H. felis*). The treatment that proved an overall most effective average eradication rate was the monotherapy with streptomycin. The combination of gentamicin and streptomycin, while second most effective in comparison to the monotherapy, proved to be less effective. This research suggests that it would be best to use a monotherapy in treating animals infected with *H. felis*.**

INTRODUCTION

H. felis is a bacterium that is strongly associated with *Helicobacter heilmannii*, the provisional name of a tightly coiled gram-negative bacteria occurring in 0.2 to 2.4% of human gastric pathologies. *H. felis*, *H. bizzozeronii* and *H. salmonis* are associated species that are naturally occurring in the stomachs of dogs and cats. The bacteria can potentially live inside of the animal for its entire lifetime which may cause gastric issues and symptoms that need veterinary care. This can affect the pet owner negatively in terms of time spent, costly medication, and visits to the veterinarian. The treatment difficulties veterinarians face is when these bacteria are abundant and present mucosal inflammation, causing clinical symptoms such as chronic vomiting and gastritis, ultimately leading to dehydration, poor appetite, abdominal pain, weight loss, and weakness (1, 2). Cats commonly acquire this easily transmitted infection shortly after they are adopted and have to be rehomed. According to the ASPCA, 42% of cats are rehomed due to costly health problems. Many times, they are denied a home as a result of their illness and are rehomed to shelters where they will infect other cats (as the bacteria is passed through saliva) and be put down if not adopted. The shift in host may be prevented by antibiotic-specific treatment to these infected animals. It is important to determine how to treat these cats and other infected animals effectively and rapidly (5).

The purpose of the study is to test the effects of various antibiotics on *H. felis*. The antibiotics that we will use during the experiment have properties that eradicate the bacteria. Antibiotics taken over a long course of treatment are known to become ineffective because the specific bacteria develop immunity as a result of the timed exposure. The mechanism of action is different between various types of antibiotics. Ampicillin and oxacillin break down bacterial cell walls by inhibiting enzyme proteins necessary for the third stage of cell wall formation. Streptomycin and gentamicin inhibit the regulation required to metabolize and repair bacterial DNA. Chloramphenicol prevents protein synthesis by diffusing through the bacterial membrane and binding to bacterial ribosomes.

Other researchers have found that *H. felis* can grown in the presence of some antibiotics, namely: vancomycin, trimethoprim, bacitracin, polymyxin B, flucytosine, and amphotericin B (4). However, an extensive number of antibiotics remain to be tested for their effect on *H. felis*. Combination therapy treatment of *H. felis* with antibiotics has not been tested either. Antibiotic combination therapy has proven effective in other studies such as Evaluation of Antibiotic Therapies for Eradication of *Helicobacter hepaticus*. In that study, a triple therapy combination of antibiotics (amoxicillin-metronidazole-bismuth (AMB) and tetracycline-metronidazole-bismuth (AMD)) proved effective in eradicating *H. hepaticus* from mice. *H. hepaticus* is a practical model when researching helicobacter-related gastric disease (3).This study will test the resistance of *H. felis* bacteria to antibiotics. Our experiment will test six different antibiotics used to treat bacterial infections, as well as two combinations of these antibiotics: ampicillin, chloramphenicol, gentamicin, oxacillin, streptomycin and tetracycline. We hypothesize that if *H. felis* resistance is tested over 24 hours with various individual antibiotics (monotherapy) and a combination of antibiotics, then the combination of antibiotics will eradicate

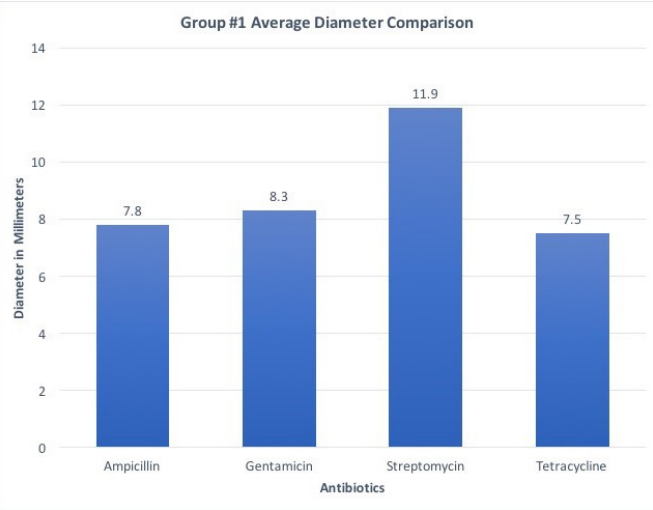

Figure 1. Group #1 Average Diameter Comparison. This result of this graph expresses the most effective mono-therapy antibiotic for eradicating *H. felis* was streptomycin in this test group. The least effective nontherapy antibiotic for eradicating *H. felis* was Tetracycline.

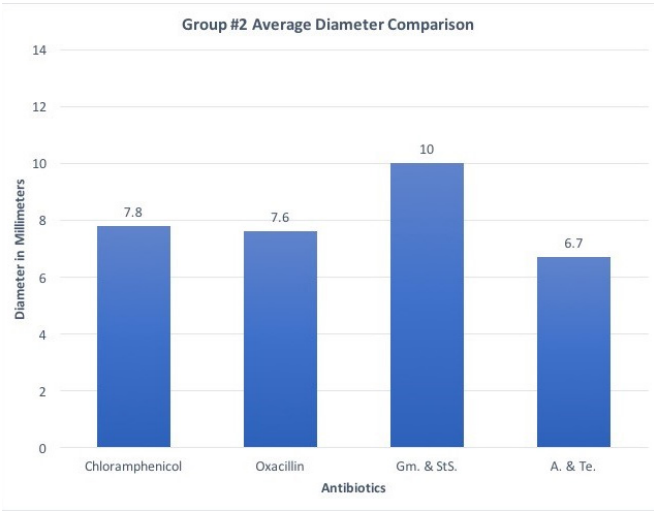

Figure 2. Group #2 Average Diameter Comparison. This result of this graph expresses the most effective combination-therapy antibiotic for eradicating *H. felis* was Gentamycin and Streptomycin in this test group. The least effective combination-therapy antibiotic for eradicating *H. felis* was Ampicillin and Tetracycline.

a higher number of bacteria.

RESULTS

We conducted this research in order to determine if a combination of antibiotics would be more effective at eradicating *Helicobacter felis* than a monotherapy of an antibiotic. In order to test antibiotic effectiveness, *H. felis* was plated on tryptic soy and subjected to the Kirby-Bauer antibiotic test with eight different antibiotic treatments. After 24 hours we measured the inhibition zones of the stand-alone monotherapy antibiotics, then decided to test the two strongest antibiotics out of the results in combination and the two weakest in combination to see if the paired antibiotics would prove effective.

The results showed that streptomycin, which proved to be the most effective antibiotic in a 24-hour period, was superior as a monotherapy as well as when combined with gentamicin. Alone, streptomycin had a resistance diameter of 11.9 mm (**Figure 1**). When combined with gentamicin, its effectiveness weakened, as it had a diameter of 10 mm (**Figure 2**). Ampicillin had a resistance diameter of 7.8 mm, while gentamicin had a diameter of 8.3 mm, and tetracycline had a diameter of 7.5 mm. Chloramphenicol had a diameter of 7.8 mm, and oxacillin had a diameter of 7.6 mm (**Figure 1**). The combination of ampicillin and tetracycline had a resistance diameter of 6.7 mm (**Figure 2**).

DISCUSSION

The results showed that streptomycin, which proved to be the most effective antibiotic in a 24-hour period was superior as a monotherapy treatment, but the data suggest it is less effective when combined with other antibiotics, as compared to the other combination treatment trials.

Our data indicate that streptomycin was effective against *H. felis* growth in vitro, suggesting that streptomycin could be a useful treatment option for veterinarians seeking to treat infected animals. It is hypothesized that the reason the antibiotic streptomycin was more effective in monotherapy rather than in combination therapy is because of the weakening effect that the antibiotic streptomycin was combined with leading to an increased amount of bacteria found with the combination therapy trials. As seen in **Figures 1** - **4** there is a direct correlation to how the bacteria performed in monotherapy and in combination therapy. The streptomycin had an average inhibition size of 11.9 mm, and the gentamicin (the antibiotic in combination therapy with the streptomycin) had an average inhibition size of 8.3 mm. The combination therapy including these two antibiotics had an average inhibition of 10 mm. This suggests that the gentamicin had a direct impact on inhibiting the antibiotic performance of the streptomycin. Similar observations were seen for the other two antibiotics that were tested in combination therapy, with the combination therapy average size being roughly equivalent to the mean of the two individual component antibiotics.

Our ability to draw conclusions from the results is limited by our experimental conditions. With more time, in a different medium and perhaps in animal tissue, we would have more biologically relevant results. We did not use the recommended tryptic soy agar liquid medium to grow the *H. felis*, instead we used another medium, lysogeny broth, to grow the bacteria. We only tested results after 24 hours. If we had extended testing, we would have had additional insight into the bacteria's infection processes. Finally, we only used an *in vitro* method to analyze our results. Alternatively an *in*

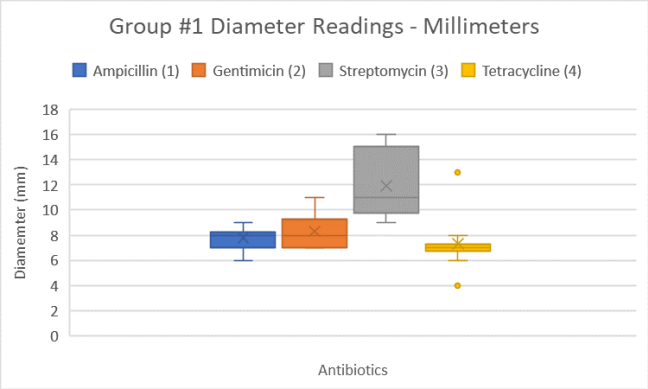

Figure 3. Group #1 Antibiotic Diameter Bar Graph Readings. This bar graph expresses the average readings that were taken from the monotherapy therapy antibiotic zone of inhibition in Group #1 on day one of testing. Recorded in millimeters (mm).

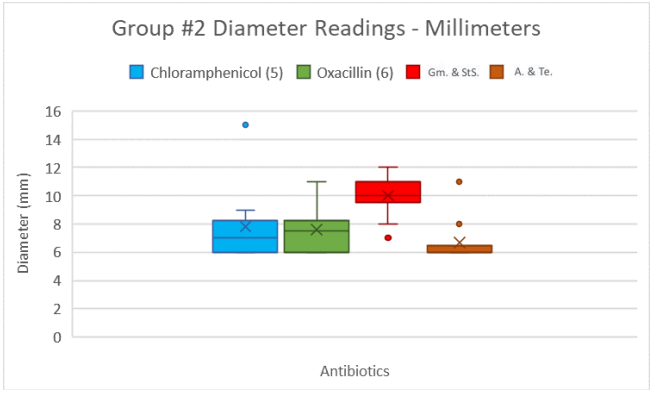

Figure 4. Group #2 Antibiotic Diameter Bar Graph Readings. This bar graph expresses the average readings that were taken from the combination therapy antibiotic zone of inhibition in Group #2 on day two of testing. Recorded in millimeters (mm).

vivo method could be tested to better simulate the biological conditions of an organism suspectable to these infections.

A previous experiment on *H. felis* called for incubation at 37ºC. We aimed to replicate this condition in our experiment; however we incubated at 35ºC due to incubator availability. We observed which petri dishes had this error directly affecting the growth of the bacteria and found no significant effect on the growth of the bacteria. Measurement error may have occurred in the experiment while measuring of diameter of the resistance spots. The spots had been scattered in various parts of the dish and this made it challenging to measure the spots with a ruler.

In the future, it may be useful to test the resistance of the *H. felis* to antibiotics other than those tested in this study, to determine their effectiveness. Using a different medium or a live animal subject in the experiments would be another area worth exploring. It may also be useful to test a probiotic after the antibiotic to assess the animal's gastric inflammation as well any decrease in symptoms. Another interesting experiment would be to see how various concentrations of each antibiotic affect the *H. felis*, in order to determine the optimal concentration and amount that could be given to a patient with this *H. felis* infection. The results of my experimentation contribute to future research in making a effort in helping animals who get *H. felis*.

MATERIALS AND METHODS

Nine hundred microliters of lysogeny broth was mixed with 100 microliters of *H. felis* to expand the sample size of the bacteria. *H. Felis* was incubated for 24 hours while shaking. Ninety microliters of distilled water were placed inside each antibiotic cell to rehydrate the various antibiotics to be used for testing. Each hydrated antibiotic solution was placed in a 2 ml tube. The streptomycin and gentamicin were combined by mixing 10 microliters of each antibiotic within a 2 ml tube. This process was repeated to combine tetracycline and ampicillin. The filter disks where then soaked in each antibiotic to apply on designated plate divisions among then tryptic soy agar plates with 5% sheep's blood plates, spread with *H. Felis*. Each dish was placed in the incubator at 35ºC for 24 hours. After the 24-hour period, we measured the diameter of the zone of inhibition in millimeters using a ruler. After testing the stand-alone monotherapy antibiotics, we tested combinations of the two strongest antibiotics and the two weakest ones to determine if these combinations of antibiotics would be effective.

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Article

Differences in Reliability and Predictability of Harvested Energy from Battery-less Intermittently Powered Systems

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SUMMARY

Solar and radio frequency harvesters serve as a viable alternative energy source to batteries in many cases where the battery cannot be easily replaced. However, energy harvesters do not consistently produce enough energy to sustain an energy consumer; thus, both the energy availability and execution of the energy-consuming process are intermittent. By simulating intermittent systems with large-scale energy demands using specifically-designed circuit models, the harvested voltage and other parameters such as the voltages across the capacitor and the load were determined. We plotted these data, for both harvested solar and harvested radio frequency energy, to make probability plots depicting the likelihood that energy will be available now given that N number of energy events have occurred. Additionally, we designated a metric as the η-factor, which was calculated from these probability plots for the solar and radio frequency data to quantify the reliability of the power source. The η-factor for harvested solar energy was statistically significantly higher than the η-factor for harvested radio frequency energy, meaning harvested solar energy was more consistently available than harvested radio frequency energy. Finally, we collected data to determine the effects on the output voltage of various obstacles between the radio frequency transmitter and receiver. We found that obstacles like metal and people caused a more pronounced drop in the amount of energy harvested when compared to other obstacles like foam or wood. Quantifying the reliability of different harvested sources would help in identifying the most practical and efficient forms of renewable energy; determining which obstacles cause the most obstruction to a signal can aid in the strategic placement of harvesters for maximum energy efficiency.

INTRODUCTION

Battery-powered devices are not suitable in many systems because of the need to frequently replace the battery. One example of this problem would be an implantable device, such as a pacemaker, whose battery would need to be replaced through surgery. Harvested energy from solar radiation, radio frequency, or other sources, such as heat, are an attractive alternative to batteries in such systems. However, since energy is not consistently available from these sources (for example, when a cloud passes in front of the sun, the energy collection is interrupted), the power availability is characterized as intermittent. There are two components to these intermittently-powered systems: the energy harvester and the energy consumer. The energy consumer uses up the energy captured by the energy harvester, and requires a designated amount of power to turn on. There is often a discrepancy between the amount of energy required to power the consumer and the amount of energy supplied to the system by the energy harvester, so the device consuming energy goes through cycles of being turned on and off **(Figure 1)**. Thus, the sporadic energy harvesting pattern leads to an interrupted, or intermittent, execution of an energy-consuming software (1). Most renewable energy sources like solar and wind power plants have an intermittent power output (2).

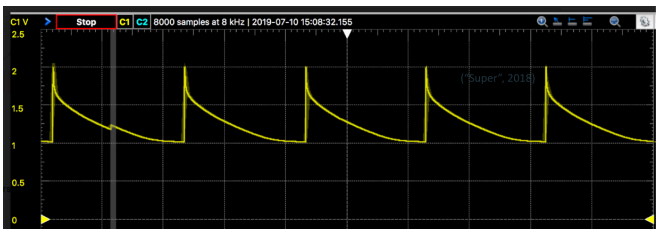

Figure 1: Graph of Voltage vs. Time for an energy consuming device. The device turns on when it reaches a threshold voltage — in this graph, the threshold voltage is approximately 2 Volts — and begins to consume power at a rate greater than the rate at which harvested energy is supplied to the circuit. When energy levels in the circuit drop enough — in this graph, when the voltage is approximately 1 Volt — the device shuts off, the energy is allowed to increase again from the harvested power supply, and the cycle repeats.

An energy event is defined as the generation of a specific amount of energy in a given time interval. In this investigation, we defined an energy event as the generation of enough energy to turn on the energy consumer (a microcontroller board), which was 2.8 Volts, over a period of five minutes. To say that N energy events have occurred is the equivalent of saying it has been 5N minutes since the energy consumer last shut off. We chose the specific time designated for an energy event in this investigation to facilitate the testing of energy available in short bursts and to allow for the data to be more easily observed when the probability of an energy event decreased toward zero. Additionally, burstiness is the property of consistency over short periods of time, and it is a feature we will be looking for in the voltage data. Finally, energy neutrality involves the introduction of an intermediate

stabilizing power supply in the circuit (3). In this investigation, the energy neutrality device was a capacitor, which is integral to the circuit. Without the capacitor, energy flowing directly from the harvester to the consumer would cause the execution of the software by the consumer to be shut off instantly in the absence of an energy event. The capacitor allowed for energy to be stored and slowly released into the consumer. This slow release through the capacitor highlights how intermittent energy sources do not provide consistent energy output to the consumer despite the stability of an energy neutrality device.

There were several goals for this investigation. The first was to model a large-scale energy harvesting system on a smaller scale using small solar harvester and radio frequency harvester units. The second goal was to test the burstiness of energy by constructing graphs of the likelihood of power availability at any given moment, given that N number of consecutive energy events had already occurred. This experiment was conducted with the hypothesis that all harvested energy will be available in short bursts, consistent over short periods of time. The third goal was to compare the reliability of different harvested sources in terms of their power availability using the η-factor. The η-factor is a calculated metric between 0 and 1 which uses the Wasserstein metric to compare the experimental energy harvesters to a random energy harvester, for which energy events are independent – this is not the case for real energy harvesters, for which energy events are conditional and dependent upon each other. We expected this experiment to show that no source will be as reliable as wall power, which has an η-factor of 1.0 but that solar energy will have a higher η-factor than radio frequency (RF) energy and thus be more reliable. The final goal was to determine how different obstacles between an RF transmitter and receiver affect the amounts of harvested energy at various distances, with the hypothesis that obstacles such as people would allow for less energy to be harvested than obstacles such as foam or wood, which were the least dense of our set of obstacles. We considered obstacles only for harvested RF energy, not for harvested solar energy; in the real world, solar energy is typically harvested without obstruction as the solar panels are placed in such a way to maximize the amount of sunlight received. In contrast, RF harvesters, which use cell towers and WiFi routers as sources, face much more obstruction, including from people, cars, or even objects around a house. These questions have great relevance at this time as the world begins to look towards renewable energy sources, such as solar, to replace fossil fuels. Investigating the patterns of energy availability and consumption allows us to predict when energy will become available or unavailable and allows for the successful scheduling of tasks or execution of processes.

RESULTS

One goal of the investigation was to test the hypothesis that harvested energy has a high amount of burstiness by constructing and analyzing graphs of the likelihood of energy availability. The idea that energy was only available in short bursts was suggested by earlier data collected in our lab, but the data collection did not occur for long enough to draw supported conclusions. To determine whether or not the energy-consuming device was turned on and how much energy was being supplied to the circuit, we recorded voltage data at various locations in the circuit, including the input, the capacitor, and the load. The data gathered for solar **(Figure 2)** and radio frequency **(Figure 3)** supported the hypothesis of the burstiness of the harvested energy, since the voltage does not increase and decrease rapidly over the majority of the graph. The correlation between probability and the number of energy events started decreasing around N = 70 **(Figure 2)**. This is consistent with the definition of an energy event designated by our lab, since 70 energy events, using the designation of 5 minutes per energy event, would be about 5.83 hours, approximately the length of time for which there was enough light outside facing the energy consumer to power it at the location this experiment was conducted. This feature is not seen in RF energy **(Figure 3)**, as the amount of harvested radio frequency energy was not dependent upon the time of day. The horizontal axes of the solar and RF probability plots contain both positive and negative values for the number of previous energy events (**Figures 2 & 3)**. Negative numbers of energy events correspond to a continuous absence of energy events. For example, when N = -40, an energy event has not occurred in the last 40 time intervals, or the last 200 minutes.

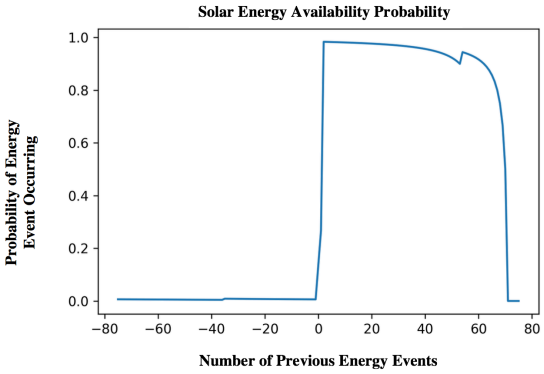

Figure 2: Probability plot for harvested solar energy. This plot displays energy occurring in short bursts. The mean η-factor is 0.8595 (n = 6). The data collection for this experiment spanned three days.

For the next objective, comparing the reliability of different harvested sources in terms of their power availability using the η-factor, we used the constructed graphs to calculate the η-factor. In regards to the hypothesis that one harvested energy source will be more reliable than another due to the fact that different energy sources are likely to have different patterns of availability over time, our findings could suggest that a certain energy source should be favored over another to allow for the most reliable execution of processes. The mean

η-factor for harvested solar power was 0.8595 and the mean η-factor for harvested radio frequency power was 0.3657, with a standard deviation of 0.0018 for the solar data and 0.0762 for the radio frequency data. We performed a student's t-test using calculations for six trials each of the solar and radio frequency experiments; the test yielded a two-tailed p-value of less than 0.0001, meaning that the difference between the η-factor for harvested solar and harvested radio frequency energy was statistically significant. The η-factors for both harvested solar and harvested radio frequency energy fall below the ideal standard of 1.0 as the η-factor for wall or battery power. For wall and battery sources, the probability of energy being available now given that any number of energy events have occurred is 1.0 **(Figure 4)** (5). The higher η-factor for solar power suggests that harvested solar energy is more reliable than harvested radio frequency.

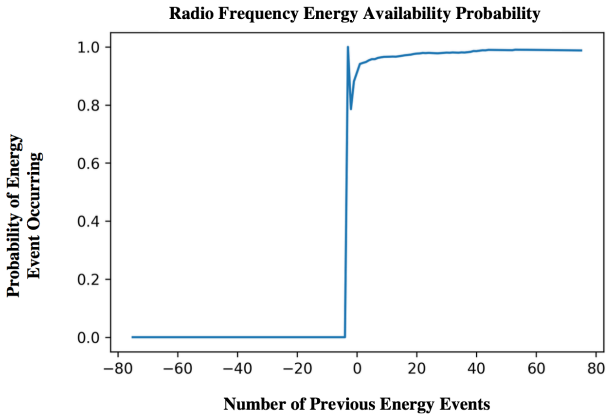

Figure 3: Probability plot for harvested radio frequency energy. This plot displays energy occurring in short bursts. The mean η-factor is 0.3657 (n = 6). The data collection for this experiment spanned two weeks.

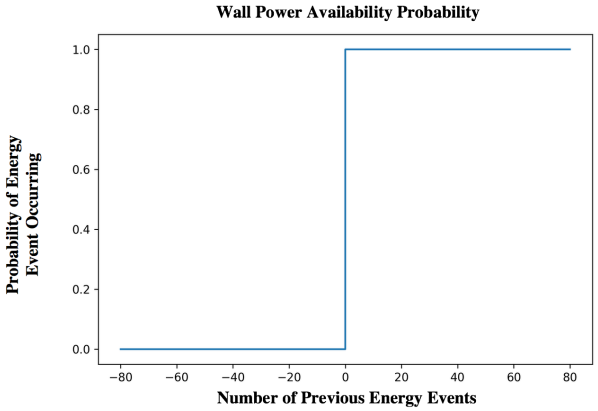

Figure 4: Theoretical probability plot for wall power. Wall power has an η-factor of 1.0 since it is not intermittent.

Finally, we placed different obstacles between a radio frequency transmitter and receiver to determine how they would affect the amounts of harvested energy; the experiments were also replicated with various distances between the transmitter and the receiver. Different obstacles are likely to have different effects on the amount of energy able to be harvested, due to density of the object, thickness, and other factors. Metal and people were the obstacles which most affected the ability of the receiver to harvest energy from the transmitter, with wood and foam having a less pronounced effect on the harvested energy **(Figure 5)**. Foam had a slightly higher voltage input value than the absence of an obstacle at a distance of 1 meter, but this difference was too small to be significant and was likely caused by random variation. Additionally, as distance increased, the received signal input decreased across all obstacles; fewer signals were received from farther away and converted into electrical energy.

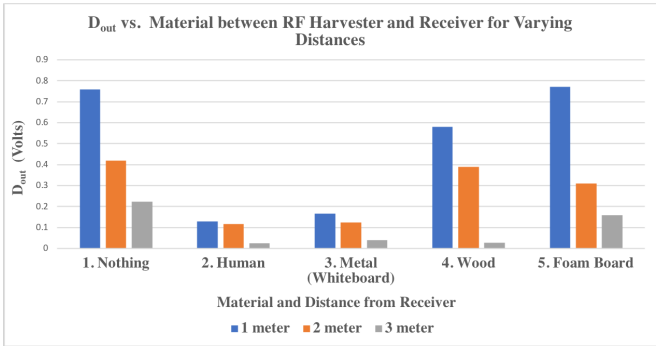

Figure 5: Graph depicting D_{out} for various obstacles and distances in the RF obstacle experiment. The D_{out} value is correlated with the radio frequency input, so higher D_{out} values correspond to higher radio frequency input.

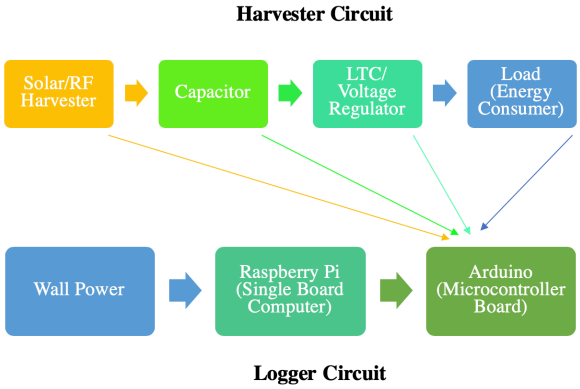

Figure 6: Diagram of circuit setups for solar and radio frequency harvesting experiments. Output voltages from each component in the harvester circuit — the harvester unit, the capacitor, the LTC, and the load — were connected to the Arduino and recorded.

DISCUSSION

The major objectives for this investigation included investigating the burstiness of energy, comparing the reliability of harvested solar and harvested radio frequency energy, and exploring the effects of various obstacles and distances on the amount of radio frequency energy able to be harvested. We constructed and analyzed graphs of the

probability of energy availability given that a certain number of energy events had occurred **(Figures 2 & 3)**, from which η-factors were calculated, addressing the first two objectives. We also plotted the average D_{out} values for different obstacles and distances in a bar graph **(Figure 5)** against a control group to compare which obstacles had the greatest effect on D_{out} values.

The solar and RF probability plots support the hypothesis of the high levels of burstiness for both harvested solar and radio frequency energy **(Figures 2 & 3)**, as the probability of an energy event occurring is relatively high after many energy events occur until the correlation stops in the case of harvested solar energy; this was due to the limited time during which there was daylight. Factors that could have influenced the display of the data include increasing the time interval t, which defines an energy event. Choosing a larger value of t may show parts of the graph well after the correlation ends for solar energy, causing probabilities in the middle to appear closer to 1 than they truly are. Choosing a smaller value of t may not show where the correlation ends, which may incorrectly suggest that the correlation does not, in fact, end. In the RF probability plot, there is a spike in probability around N = -1 **(Figure 3)**, meaning that if in the previous time interval there was no energy event, then the probability of an energy event occurring now is extremely high. This suggests that when a person walks in front of the sensor, they cause the absence of a single energy event, but they are not likely to cause the absence of a second energy event; in other words, most people walk by the sensor rather than standing in front of it. Thus, when considering human interference in real-world RF-harvesting situations, we believe they do not tend to stand in place and obstruct the signal for extended periods of time. Factors that may have influenced the data collection include the sensor recording the presence of a person when there was not a person, or failing to record the presence of a person when there was one. Finally, the Arduino could only record voltages to two decimal places, restricting the precision of the data analysis. These results are significant as the burstiness of energy will allow for more ease in the process of scheduling tasks to be executed.

The calculations of the η-factors and the results of the student's t-test suggest that harvested solar power is more reliable than harvested radio frequency power, supporting our hypothesis. One potential reason for the higher standard deviation for the radio frequency η-factor could be the increased variance among trials in the patterns of people passing in front of a sensor over a given time, compared to the more stable pattern of light reaching a solar panel. We performed calculations for harvested radio frequency energy using data which spanned a longer period of time than the solar data collection – a difference of about two weeks for RF energy versus three days for solar energy. The rationale behind this was that the absence of radio frequency energy events is less frequent than the absence of solar energy events, so more data needed to be analyzed to get a good overall picture for radio frequency energy. The same factors which could have caused error in the first experiment apply here – the reliability of the sensor and the accuracy of the Arduino. These results are significant as harvested solar energy may be a more suitable alternative to harvested radio frequency energy in terms of reliability and predictability.

Our data also suggests that people and metal most obstruct the radio frequency signal from being received and converted into electrical energy **(Figure 5)**. Thus, people were used to obstruct the signal in the radio frequency experiment in order to induce the absence of an energy event. Factors that could have influenced these results include the presence of multiple objects between the transmitter and receiver, such as a hand holding the foam board, and again, the accuracy of the Arduino for recording voltages. These results are relevant to the real-world application of harvested radio frequency energy – from WiFi routers, cell towers, and more – by suggesting which obstacles are more likely to cause the absence of an energy event and should be considered and avoided when choosing a location for a potential radio frequency energy harvester.

In regards to the goal of accurately modeling large-scale solar and radio frequency energy harvesting systems, there are some aspects of the models which translate better to the real world than others. For example, the placement of the solar panels in a location which maximizes the amount of sunlight received and the fact that a solar panel can only face towards a single direction are two characteristics of the model that reflect practical circumstances. However, for the radio frequency experiment, the model fails to account for the real-life conditions of multiple obstacles or static obstacles, which could decrease the likelihood of energy events by allowing for less energy to be successfully harvested.

Future experiments may involve conducting the same experiment for different forms of harvested energy, including piezoelectric energy which is the generation of electrical energy in response to mechanical pressure. Piezoelectric energy can be harvested in many forms, and another possible avenue of exploration would be the differences in amounts and reliability of harvested energy between different types of piezoelectric energy, including energy harvested from mechanical stress inside a person's shoe or from mechanical stress on a sidewalk or tile. Additionally, the solar experiment could be replicated under different weather conditions; if there were less sunlight available, it would be expected that energy events would occur less frequently, and if there were more sunlight available, energy events would occur more frequently. One interesting avenue of exploration with obstacles is including them for harvested solar energy in addition to harvested radio frequency energy. If obstruction were considered for harvested solar energy, the effect on voltage would likely be correlated with the opacity of the object obstructing the sunlight. Also, different densities and thicknesses of the obstacles could be tested. Finally, these experiments could be replicated using a different frequency

for the radio frequency transmitted and the results could be compared to those of this experiment.

As we look to replacements for fossil fuels in this era of climate crisis, we should keep in mind the reliability of harvested energy sources. Harvested solar energy was found to be more reliable than harvested radio frequency energy, and we should consider this when deciding which types of renewable energy we want to invest in and implement on a large scale. When placing these harvesters, particularly radio frequency harvesters, we should attempt to place them in a way that minimizes obstruction from different objects, especially people and metal. In this way, we can maximize energy efficiency. Through harvesting energy from common and everyday sources like the sun and radio frequency signals, we take the first steps towards ensuring a more sustainable and energy-efficient future.

MATERIALS AND METHODS

The setup for both the solar and the radio frequency experiments for which the probability plots were constructed involved two main circuits **(Figure 6)**. The first, designated as the harvested circuit, captured either sunlight or a transmitted signal and converted it into energy. There were four main components to this circuit: the solar or radio frequency harvester unit, the capacitor to store energy and provide the circuit with more stability, the Load Tap Changer (LTC) , and the load, or the consumer. In this experiment, the load was an MSP-430 device (Texas Instruments) which was constantly running an energy-consuming software and required 2.8 Volts to turn on. The purpose of the LTC in the circuit was to ensure that given a certain amount of energy input, the amount of energy output would be 3.3 Volts; this simply amplified the input voltages so that the absence of sufficient energy would be more pronounced in the voltage recordings. We recorded voltage data at various locations in this circuit, including across the energy harvester, which captured the energy from the sun or from the radio frequency transmitter, and across the energy consumer. The voltages were recorded and printed out using a Python 3 program, an Arduino Uno (SparkFun Electronics), and a Raspberry Pi 3 (Raspberry Pi Foundation). The weather on the days the experiment was conducted is important to consider when dealing with solar data, so a light sensor was also used to record the full-spectrum, infrared, and visible light levels; part of the Python program retrieved real-time weather data from weather.com, including temperature and UV index. The weather on all three days during which the experiment was conducted was sunny with minimal clouds. The harvester was placed directly across from a window facing west, so that the system tended to reach peak energy in the afternoon, when the most sunlight was available to it.

For the radio frequency experiment, we used an infrared sensor to record the presence or absence of people; a person walking in front of the sensor, and thus blocking the signal, was considered as the absence of an energy event. The second main circuit, the logger circuit, was constructed to record voltages at each point in the harvester circuit. It included wall power connected to a Raspberry Pi 3 and an Arduino Uno. We wrote an Arduino program and a Python program to print out and save the recorded voltages into a .csv file. We then analyzed the data, calculated the η-factors, and constructed probability plots using a different Python program in Jupyter Notebook.

The setup for the obstacle experiment using a radio frequency harvester involved the same circuit setup as the other radio frequency experiment, but without the use of the infrared sensor. A Python and an Arduino program were used to record the voltages at various points in the circuit. Data were collected over a span of two minutes each for four different obstacles – metal, wood, person, and foam – over three different distances – 1 meter, 2 meters, and 3 meters. Note that the person used in this particular experiment was instructed to stand between the transmitter and harvester for two minutes; this experiment was conducted to establish that when a person is between the RF transmitter and receiver, they cause the absence of an energy event. We then used this fact in the radio frequency event, where we recorded when people walked in front of the sensor and considered it as the absence of an energy event. The voltages for this obstacle experiment were recorded from the D_{out} pin on the MSP-430, which is directly related to the amount of radio frequency input received. The absence of an obstacle between the transmitter and the receiver served as a control to which the other voltage values could be compared. We saved these data in a .csv file using a Python and Arduino program and further analyzed and graphed the data using Microsoft Excel. The approximate densities and thicknesses of the obstacles used were recorded **(Table 1)**. We approximated the density of a human at 1.01 grams per cubic centimeter (4).

Approximate Thickness and Density of Obstacles		
	Thickness (cm)	Density (g/cm^3)
Human	25	1.01
Metal Whiteboard	2.0	0.85
Wood	2.0	0.70
Foam	0.3	0.13

Table 1: Approximate thicknesses and densities of obstacles used in experiment. The thicknesses were measured and densities were calculated using measurements of mass and volume. The thickness measurement for a human is very approximate as it is harder to measure with precision.

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Article

Trajectories between Cigarette Smoking and Electronic Nicotine Delivery System Use among Adults in the U.S.

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SUMMARY

Smoking causes six million deaths annually worldwide. There are controversies regarding the use of electronic nicotine delivery systems (ENDS) to help smokers quit. The purpose of this study was to examine trajectories from prior ENDS use to current cigarette use among adults in the United States. We used National Cancer Institute public data from 2018 to build two statistical models. For model 1, we examined the association between prior ENDS use (vs. never ENDS use) and current cigarette use. For model 2, we examined the association between ENDS use (current and former vs. never) and continuing to smoke among ever smokers. For both models, sociodemographic covariates included age, race and ethnicity, sex, and income and incorporated survey weights. The Health Information National Trends Survey (HINTS) data set included 3,437 participants representing all 245,360,828 people 18 and over in the United States. Compared with those who were not prior ENDS users, prior ENDS users had about 10 times the odds of being current cigarette smokers (AOR=9.74, 95% CI=5.82, 16.31). Additionally, among ever smokers, compared with those who were never ENDS users, current ENDS users were significantly more likely to be current smokers (AOR=5.69, 95% CI=2.46, 13.16). Thus, ENDS use was strongly associated with later cigarette smoking, and smokers who use ENDS were more likely to continue smoking than those who did not use ENDS. These results underscore concerns regarding ENDS and question whether ENDS represent a valuable tool to help smokers quit.

INTRODUCTION

Smoking causes six million deaths annually worldwide. This is true despite the fact that many effective means of quitting smoking exist, including nicotine replacement therapy such as the nicotine patch and nicotine gum. Effective pharmacologic means include bupropion and varenicline. However, even with these tools, smoking cessation is low (1, 2).

Therefore, other proposed tools to help smokers quit are electronic nicotine delivery systems (also called e-cigarettes or ENDS). ENDS, introduced to the market in 2003, use electricity and heat to convert liquid nicotine, propylene glycol, flavorings such as strawberry or chocolate, and sweeteners such as glycerin into an inhalable aerosol (3). ENDS do expose users to toxins such as tar, carcinogens, and heavy metals (4). In addition, there have been recent high-profile deaths related to the use of ENDS (5). However, compared with cigarettes, ENDS seem to expose users to lower amounts of these chemicals on average (4). Therefore, some experts suggest that switching cigarette smokers to ENDS can help them transition away from smoking entirely (6).

However, other studies show that ENDS may cause non-smokers to transition to traditional cigarettes. In these studies, even people who initially had very low risk of cigarette smoking ended up being smokers after experimenting with ENDS (7–9). Importantly, these studies focused on adolescents and young adults.

One reason for these contradictory results may be that these studies examined different populations. For example, studies focusing on the trajectory from experimenting with ENDS to becoming traditional cigarette users have generally involved adolescents (7, 10). However, studies examining the trajectory away from cigarette use to ENDS use have generally involved specific populations of adults (11). So, it would be useful to examine trajectories in the same population of individuals. In addition, most studies related to ENDS have focused on adolescents, and fewer studies have involved adults for whom cessation is a priority.

Therefore, the purpose of our study was to examine trajectories from prior ENDS use to current cigarette use in an adult population. Because we focused on a population of adults, and because of the studies described above that showed efficacy of ENDS for cessation, we hypothesized that former (vs. never) ENDS use would be significantly associated with lower current cigarette use (H1). However, because there is little indication that adults transition from ENDS to traditional cigarettes, we also hypothesized that there would be no significant association between prior ENDS use and continuing to smoke cigarettes among ever smokers (H2). We hoped that examining these data would help public health and medical professionals to understand the relationship between use of ENDS and traditional cigarettes.

RESULTS

We accessed the public use data set for the Health Information National Trends Survey (HINTS) Wave 5, Cycle 2

		Current Cigarette Use[a]	
		OR (95% CI)	AOR (95% CI)[b]
ENDS Use[c]	Never	1.00	1.00
	Former	**6.27 (3.93, 9.99)**	**9.74 (5.82, 16.31)**
Age, years	18-34	1.00	1.00
	35-49	**2.77 (1.53, 5.00)**	**4.79 (2.41, 9.51)**
	50-64	**2.87 (1.62, 5.09)**	**6.12 (3.08, 12.18)**
	65-74	1.15 (0.63, 2.11)	**2.28 (1.08, 4.84)**
	75+	0.90 (0.44, 1.85)	1.81 (0.73, 4.46)
Sex	Male	1.00	1.00
	Female	**0.59 (0.42, 0.84)**	**0.56 (0.37, 0.86)**
Racial/ethnic category	Caucasian	1.00	1.00
	African-American	1.66 (0.89, 3.09)	1.30 (0.67, 2.54)
	Hispanic or Latino	1.16 (0.65, 2.09)	0.73 (0.39, 1.37)
	Asian	0.91 (0.41, 2.04)	0.52 (0.15, 1.75)
	Other[d]	1.71 (0.58, 5.03)	1.81 (0.49, 6.61)
Annual Household Income	Less than $20,000	1.00	1.00
	$20,000 to $34,999	**0.45 (0.25, 0.78)**	**0.55 (0.27, 1.10)**
	$35,000 to $49,999	**0.51 (0.28, 0.90)**	**0.52 (0.28, 0.98)**
	$50,000 to $74,999	**0.39 (0.22, 0.72)**	**0.36 (0.18, 0.72)**
	$75,000 or more	**0.39 (0.22, 0.67)**	**0.32 (0.17, 0.57)**

Table 1: Trajectories from prior cigarette use to current ENDS use and prior ENDS use to current cigarette use among adults in the United States, 2018.
Abbreviations: AOR, Adjusted Odds Ratio. CI, Confidence Interval. ENDS, Electronic Nicotine Delivery Systems. OR, Odds Ratio.
Note: Bold values indicate statistical significance at the level of α = 0.05 for logistic regression analyses.
[a] Current use was defined as use in the past 30 days.
[b] Adjusted for all variables in the table.
[c] Prior use was defined as use in the past but not in the most recent 30 days.
[d] The other category for race and ethnicity included American Indian, Alaska Native, Native Hawaiian, Other Pacific Islander, and mixed race.

(12). HINTS is an ongoing survey conducted by the National Cancer Institute (NCI). This survey, which focuses on how people get information related to cancer, has been conducted since 2003. It assesses a nationally-representative sample of United States (US) residents ages 18 and above. Wave 5, Cycle 2 was conducted in 2018. HINTS provides survey weights to help researchers adjust the sample to reflect the whole population of the United States with regard to age, gender, educational attainment, race, ethnicity, and census region.

For the purpose of this study, we focused on the variables for ENDS and cigarette use. Both variables were categorized by HINTS as current, former, or never. We also used sociodemographic variables including age, race and ethnicity, biological sex, and household income.

The final HINTS sample included 3,437 participants representing 245,360,828 people ages 18 and above. When incorporating survey weights, the sample was 50.8% female. Participants were 64.8% Caucasian, 10.8% African-American, 16.0% Hispanic or Latino, 5.2% Asian, and 3.3% in other racial and ethnic categories. The average age of the sample was 48.8 with a standard deviation of 17.3 years. When accounting for survey weights, cigarette smoking status was 15.8% current, 20.6% former, and 63.6% never. Also accounting for survey weights, ENDS status was 3.6% current, 13.2% former, and 83.2% never.

For our main analyses, we built two statistical models. For model 1, we examined the association between prior ENDS use and current cigarette use. For this analysis, we omitted current ENDS users in order to compare prior ENDS users to never ENDS users. For model 2, we wanted to test whether use of ENDS was related to continued smoking among smokers. Therefore, we examined the association between ENDS use (never, former, or current) and continued cigarette use (as opposed to being a former user). For both models, we used all sociodemographic covariates and incorporated survey weights.

In the analysis that included all variables, compared with those who were never ENDS users, those who were former ENDS users had about 10 times the odds of being current cigarette smokers (AOR=9.75, 95% CI=5.82, 16.33). Therefore, H1 was not supported. Certain sociodemographic

		Remain Cigarette Use[a]	
		OR (95% CI)	AOR (95% CI)[b]
ENDS Use[c]	Never	1.00	1.00
	Former	**3.27 (2.02, 5.28)**	**3.01 (1.69, 5.34)**
	Current	**5.33 (2.54, 11.20)**	**5.69 (2.46, 13.16)**
Age, years	18-34	1.00	1.00
	35-49	1.08 (0.54, 2.16)	1.56 (0.70, 3.50)
	50-64	0.83 (0.43, 1.61)	1.44 (0.66, 3.12)
	65-74	**0.24 (0.12, 0.47)**	**0.38 (0.16, 0.88)**
	75+	**0.17 (0.08, 0.38)**	**0.27 (0.10, 0.74)**
Sex	Male	1.00	1.00
	Female	**0.61 (0.43, 0.88)**	**0.58 (0.37, 0.90)**
Racial/ethnic category	Caucasian	1.00	1.00
	African-American	**2.47 (1.19, 5.13)**	1.75 (0.80, 3.83)
	Hispanic or Latino	**2.57 (1.37, 4.79)**	1.50 (0.77, 2.94)
	Asian	1.48 (0.54, 4.09)	0.95 (0.28, 3.20)
	Other[d]	2.72 (0.99, 7.49)	1.48 (0.49, 4.56)
Annual Household Income	Less than $20,000	1.00	1.00
	$20,000 to $34,999	**0.44 (0.23, 0.83)**	0.51 (0.24, 1.10)
	$35,000 to $49,999	0.63 (0.34, 1.17)	0.59 (0.30, 1.16)
	$50,000 to $74,999	**0.34 (0.19, 0.64)**	**0.36 (0.18, 0.74)**
	$75,000 or more	**0.38 (0.22, 0.67)**	**0.29 (0.15, 0.56)**

Table 2: Factors related to remaining a cigarette smoker among ever smokers, 2018.
Abbreviations: AOR, Adjusted Odds Ratio. CI, Confidence Interval. ENDS, Electronic Nicotine Delivery Systems. OR, Odds Ratio.
Note: Bold values indicate statistical significance at the level of α = 0.05 for logistic regression analyses.
[a] Current use was defined as use in the past 30 days.
[b] Adjusted for all variables in the table.
[c] Prior use was defined as use in the past but not in the most recent 30 days.
[d] The other category for race and ethnicity included American Indian, Alaska Native, Native Hawaiian, Other Pacific Islander, and mixed race.

variables were related to cigarette and ENDS use in this model. Compared with people 18-34, those ages 35-74 were significantly more likely to be current cigarette smokers (**Table 1**). Compared with males, females were about half as likely to be current cigarette smokers (AOR=0.56, 95% CI=0.37, 0.86). Wealthier individuals were less likely to be cigarette smokers (**Table 1**). There were no significant associations between race and ethnicity and current cigarette use.

Among ever smokers, when covariates were included, compared with those who were never ENDS users, smokers who were former ENDS users had about 3 times the odds of remaining as cigarette smokers (AOR=3.01, 95% CI=1.70, 5.35). Furthermore, compared with those who were never ENDS users, those who were current ENDS users had about 6 times the odds of remaining as cigarette smokers (AOR=5.72, 95% CI=2.47, 13.24). Therefore, H2 was not supported. In this model, remaining a cigarette user was also associated with lower odds of being older, female, and wealthier (**Table 2**). There were no significant associations between race and ethnicity and remaining a cigarette smoker.

DISCUSSION

This nationally representative study that used NCI HINTS data had two major findings. First, this study found that being a former ENDS user, as opposed to never using ENDS, was strongly associated with being a current cigarette smoker. Second, we found that, among ever cigarette smokers, compared with being a never ENDS user, being a former or current ENDS user was strongly associated with continuing to smoke cigarettes.

The former finding suggests that initially using ENDS and then transitioning to cigarettes is a common pattern. There are multiple reasons why we might see this pattern. First, ENDS expose users to nicotine, a highly addictive drug, in a palatable form. Once users are sensitized to nicotine and build tolerance, they may seek it out in stronger forms, including cigarettes. Second, once users are accustomed to the behavioral aspects of ENDS use—such as holding the implement and bringing it to their lips—they are more likely to transfer those same habits to cigarette smoking. Third, ENDS use may come along with social situations that may pressure users into cigarette smoking. This pattern of ENDS

use transitioning to cigarette use has been found in prior studies (7, 9, 10). However, this study adds to that body of work because of its focus on adults. Most prior studies have focused on adolescence, but our sample included young adults, middle-aged individuals, and the elderly.

These findings support the recent steps taken by the Food and Drug Administration (FDA) to regulate ENDS (13). For instance, in 2016 the FDA made it illegal to sell ENDS products to people under 18, and they enforce this policy by issuing warning letters and penalizing stores that sell these products to minors. In addition, the FDA conducts inspections of ENDS stores and manufacturers. The FDA also requires labeling of ENDS products with ingredients and a nicotine warning.

We also found that, among ever cigarette users, there was a strong relationship between being an ENDS user and remaining a cigarette smoker. This suggests that ENDS may not represent a cessation tool to quit smoking. In fact, their use may inhibit the ability to stop smoking. The data around ENDS as a cessation tool have been mixed. One randomized, controlled trial conducted in New Zealand, found that ENDS were no better than the nicotine patch for cessation (6). More recently, however, a study in England found that ENDS was better than nicotine replacement therapy for cessation (14). However, the benefit was relatively weak, and this study did not compare ENDS to more established cessation tools such as bupropion or varenicline. However, a randomized, controlled study recently showed those who were assigned to ENDS use and counseling for cessation were far less successful than those assigned to counseling only. In particular, 10.1% of the smokers who were randomized to use e-cigarettes for cessation had quit smoking after 6 months compared to 26.6% of smokers who were assigned to no specific treatment (15).

Our findings were most consistent with this latter study. There are several reasons why use of ENDS may counterintuitively reduce the likelihood of cessation. One reason is that they may propagate a cycle of nicotine addiction instead of encouraging complete cessation. Unlike nicotine patches, for instance, which are simply stuck on the body, ENDS use involves actions and patterns similar to traditional cigarette use. This can include a similar daily routine as smoking, such as using the implement once an hour. It also involves holding onto and dragging from the implement in a similar way. Indeed, studies show that many ENDS users become dual users of ENDS and cigarettes (16).

The most important limitation of this study is that, because of the study design, it cannot determine causality. For example, just because someone is a former ENDS user and now smokes cigarettes, this does not mean that the initial ENDS use necessarily caused the smoking. It will be useful for future studies to use longitudinal designs to more carefully examine trajectories to help us determine causality. Another limitation was the wording of the tobacco and nicotine questions in the HINTS survey. For example, for the question determining cigarette use, participants were asked if they had smoked 100 cigarettes in their lifetime, but the ENDS question asked if they had used an ENDS product at all. This was due to the fact that ENDS is still a newer behavior with less defined assessment tools. Over time, large studies such as HINTS likely will develop more standardized methods of measuring ENDS use.

In any large, self-report study, there is the risk of dishonesty. Because the HINTS survey did not use biochemical validation, we cannot determine the validity of participants' responses. However, it is unlikely that the participants were dishonest, because ENDS and cigarettes are legal, and the study was conducted anonymously. Still, future studies might employ biochemical validation to confirm the integrity of the participants' responses.

In conclusion, despite these limitations, this study helps add to the literature because it showed that there are associations between being a former ENDS user and a current cigarette smoker, as well as showing that there is a strong association between ENDS use and continuing to smoke cigarettes among ever smokers. It is important to continue researching this topic using both qualitative and quantitative methodologies.

MATERIALS AND METHODS

Data Source and Participants

For this study we conducted a secondary analysis of HINTS data. The HINTS survey is a project of the National Cancer Institute. It has been conducted 13 times since 2003. HINTS uses a cross-sectional model to survey participants about topics related to cancer and health information. For this study we used HINTS 5 Cycle 2, collected from January through May of 2018 (12).

HINTS 5 Cycle 2 used a paper questionnaire mailed to random houses from the United States Postal Service list of residential houses. Then, the adult the survey was given to was selected using the next birthday method. This method chooses the next person to have a birthday in that household. HINTS only included adults. This means that it excluded people under 18 years of age. It also excluded institutionalized individuals and military personnel serving overseas.

HINTS created survey weights to adjust the sample to reflect the whole population of the United States. For example, if the proportion of females in the sample was smaller than the proportion of females in the US, the weights would help overweight the females' responses. The survey weights also adjusted for non-response. Demographic variables that were used to create the survey weights included age, gender, educational attainment, race and ethnicity, and census region.

The HINTS study received Institutional Review Board (IRB) approval from the National Institutes of Health. For the current analysis, we did not require additional IRB approval because we only conducted secondary analyses and did not collect additional primary data.

Measures

HINTS categorizes people into being current, former, or never smokers. In order to define the participants' smoking status, they were asked two questions: "Have you smoked at least 100 cigarettes in your entire life?" (with responses of yes and no) and "Do you now smoke cigarettes every day, some days, or not at all?" (with responses of everyday, some days, or not at all). Current smokers were defined as those who answered either "everyday" or "some days" to the latter question. Former smokers were defined as those who answered "yes" to having smoked 100 cigarettes in their lifetime and "not at all" to the latter question. Never smokers were defined as those who answered "no" and "not at all" respectively.

In order to define the participants' ENDS use status, they were also asked two questions: "Have you ever used an e-cigarette, even one or two times?" (with responses of yes and no) and "Do you now use an e-cigarette every day, some days, or not at all?" (with responses of everyday, some days, or not at all). Current ENDS users were defined as those who answered either "everyday" or "some days" to the latter question. Former ENDS users were defined as those who answered "yes" to having tried ENDS before and "not at all" to the latter question. Never ENDS users were defined as those who answered "no" to the former question and "not at all" to the latter question.

We used four demographic variables: age, gender, race, and annual household income. HINTS categorizes age into five predetermined categories: 18-34, 35-49, 50-64, 65-74, and 75+. For gender, respondents self-reported as being either male or female. For the question regarding race and ethnicity, there were six options, and participants were allowed to select as many as they liked: Hispanic, White, African-American or Black, Native American or Native Alaskan, Asian, and Pacific Islander. We collapsed these into five mutually-exclusive categories: Hispanic, White, Black, Asian, as well as "other," which included Native American, Native Alaskan, Pacific Islander, and mixed race. We created this "other" category because there were too few American Indians and Pacific Islanders to have their own categories, and we did not want our models to be unstable due to small cell sizes. For household income, participants were asked "What is your combined annual income, meaning the total pre-tax income from all sources earned in the past year?" They responded in 5 categories: Less than $20,000, $20,000 to $34,999, $35,000 to $49,999, $50,000 to $74,999, and $75,000 or more.

Analysis

To determine the association between being a former ENDS user and a current cigarette smoker we used logistic regression. This is because our dependent variable, current cigarette smoking, is dichotomous. Our primary independent variable for this analysis was former (vs. never) ENDS use. For this analysis we did not include current ENDS users because we wanted to directly compare former ENDS use to never ENDS use.

To determine the association between being a current or former ENDS user and continuing to smoke we also used logistic regression. For this analysis we did not include never cigarette users so current cigarette use effectively meant continuing to smoke.

We included in our primary models all relevant demographics, including age, gender, race, and annual household income. We included survey weights in all of our primary models. We also conducted secondary models to examine the robustness of our results. For example, we conducted analyses without survey weights. We also conducted logistic regression analyses that only included control variables that were significantly associated with the outcome variables at $P<0.15$. Because the results of these secondary analyses were similar to the primary analyses, we only include results from primary analyses in this report.

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Article

The Effects of Ezetimibe on Triglyceride and Alanine Transaminase Reduction in *Drosophila Melanogaster* Model of Nonalcoholic Fatty Liver Disease (NAFLD)

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SUMMARY

Nonalcoholic Fatty Liver Disease (NAFLD) is a condition where a surplus of triglycerides or fat are present in the liver. The mechanisms leading to NAFLD are variable and poorly understood; however, it is known that high cholesterol, obesity, poor diet, and diabetes are risk factors for fatty liver disease. NAFLD has been previously induced in *Drosophila melanogaster* through the administration of a coconut oil-based diet, establishing this as a useful model to study the disease and possible treatments. Researchers monitor the progression of the disease through measuring triglyceride levels or alanine transaminase levels, two markers of NAFLD. In this study, ezetimibe, a cholesterol lowering drug, was used to treat flies modeling NAFLD. It was hypothesized that if ezetimibe was tested in a model of NAFLD in *Drosophila melanogaster*, ezetimibe would effectively lower triglyceride and alanine transaminase levels, two markers of the disease. Flies were held in vials with either a high fat diet or a control diet and were then transferred to control diet vials with or without the drug treatment. Flies were assayed every day over a period of five days and two datasets were collected. Compared to the coconut oil-fed flies that were transferred to the control medium, the flies transferred to the control medium treated with ezetimibe showed a decrease in their triglyceride and alanine transaminase level.

INTRODUCTION

Nonalcoholic Fatty Liver Disease (NAFLD) affects between 80 and 100 million individuals in the United States and is approximated to affect as many as one billion people worldwide (1). It is very common in patients with obesity and has the most cases per year of any liver disease in the United States. Currently, there is no primary treatment for NAFLD and recommended treatments include diet and lifestyle changes, such as increased exercise and the avoidance of certain foods that are high in fat (2).

NAFLD is linked to many other disorders and conditions and has proven to be more common in patients with already present metabolic disorders (3). When numerous metabolic disorders occur concurrently, it is called metabolic syndrome. Metabolic syndrome usually consists of dyslipidemia, central obesity, raised blood pressure, and insulin resistance (1). Insulin resistance is one of the hallmarks of NAFLD and heavily influences the pathogenesis of the disease. It is also a key factor in the transformation of NAFLD to nonalcoholic steatohepatitis, or NASH. Marchesini et al. (1999) were the first to point out the important partnership between insulin resistance and NAFLD, showing that adult patients with NAFLD are sensitive to insulin and have similarly damaged hepatic glucose production as patients with overt type 2 diabetes (4). Steatosis, an important part of NAFLD, happens when the speed of hepatic fatty acid genesis is higher than the speed of fatty acid export and oxidation. This imbalance forms a surplus amount of intrahepatic triglyceride, which creates a disproportion in the complex metabolic system. The existence of steatosis in the liver creates a network of detrimental alterations to glucose, lipoprotein, and fatty acid metabolism (5).

The main marker used to screen for NAFLD is alanine transaminase (6). Also known as alanine aminotransferase (ALT), ALT is an enzyme that is mainly found in the kidney and liver. High levels of ALT have been heavily linked with increased liver damage (6). In most cases of NAFLD, ALT levels are increased to above normal quantities, and therefore, have been used as a marker for NAFLD (6).

There is no standard treatment for NAFLD, so there is a need to find a drug that might work to reduce symptoms or even cure the disease. Ezetimibe is a drug that can be used alone or in conjunction with lifestyle modification to lower the amount of cholesterol and other fatty content in the blood and is currently approved to treat atherosclerosis (7). Ezetimibe impedes cholesterol absorption from the intestinal lumen into enterocytes by targeting a sterol transporter called Niemann-Pick C1-like 1 protein (NPC1L1) (7). Human NPC1L1 is highly overexpressed in the liver and has been linked with hepatic accumulation of cholesterol, similar to the pathology of NAFLD. In the human clinical trials ezetimibe was shown to provide a powerful reduction in total cholesterol levels, triglycerides, and low-density lipid (LDL) cholesterol. One study using human subjects approved ezetimibe being used for patients with high cholesterol; however, studies using ezetimibe to treat NAFLD

have so far been inconsistent (7).

To model NAFLD in *D. melanogaster*, we fed them a high-fat diet by mixing coconut oil into the standard medium. Using coconut oil in *D. melanogaster* is an established method of inducing diseases associated with an accumulation of fat, such as heart disease and obesity (8). The main benefit of using *D. melanogaster* as a model organism is that it shares many basic biological process with humans. We chose to use a diet where coconut oil makes up thirty percent of the medium, as opposed to other methods, because it has shown to be a cheap and effective method to induce NAFLD.

In this study, we used a *D. melanogaster* model of NAFLD to study the effect of treatment with ezetimibe would have on two markers of the disease, triglyceride levels and ALT levels. The purpose of the experiment was to determine whether ezetimibe could be an effective treatment for NAFLD. We hypothesized that if ezetimibe is used against NAFLD in flies, it will lower triglyceride and ALT levels, effectively lowering two markers of the disease. Flies given the coconut oil treatment should have increased triglyceride and ALT levels compared to the control, and we would expect that flies transferred to the ezetimibe treatment would have a decrease in these markers, regardless of their starting condition.

RESULTS

To determine the effect of ezetimibe on flies modeling NAFLD, we prepared parallel *Drosophila* populations in vials of control medium and high fat medium, which was supplemented with coconut oil. After three days, flies in both vials were either transferred to tubes with control medium supplemented with ezetimibe or tubes with control medium (**Figure 1**). Flies were assayed, or tested, every day over the span of five days, and two curves of data were created: one that measured triglyceride levels and another that measured ALT on which after the conclusion of the experiment, independent T-tests were ran. Compared to the coconut oil-fed flies that were transferred to the control medium, the coconut oil-fed flies transferred to the medium treated with ezetimibe showed a decrease in their triglyceride and ALT levels (**Figures 2-3**). After three days on the high-fat, coconut oil diet, flies showed an 88.04% increase in milligrams of triglycerides per mL. The increase can

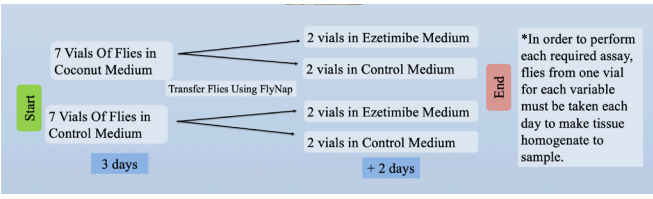

Figure 1. Procedure flow chart. Flies were either kept in coconut oil or control medium for a three-day period and were then transferred to either an ezetimibe or control medium for a two-day period, creating four variables. Flies from each variable were assayed over the course of the five-day period.

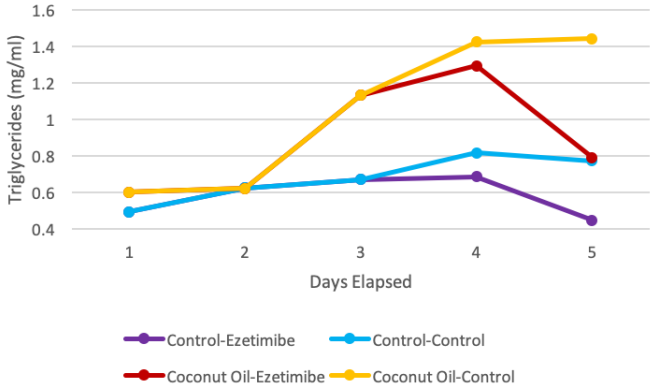

Figure 2. Triglyceride levels measured over a period of five days. The average levels of triglycerides (mg/mL) in D. melanogaster were measured over five days. Five flies for each variable (Control-Ezetimibe, purple; Control-Control, blue; Coconut oil-Ezetimibe, red, and Coconut oil-control, yellow) were used for each measurement. Error bars represent a 95% confidence interval.

be seen using the triglyceride colorimetric assay, with the rapid increase in triglyceride levels observed in the coconut oil-fed flies in the first three days (**Figure 2**). Flies transferred to the control media supplemented with ezetimibe showed a 38.40% decrease after 48 hours, while flies transferred to control media alone showed a 27.40% increase. After three days on a high fat diet, flies showed a 20.76% increase in units of ALT per mL. When these flies were then transferred to control media or control media supplemented with ezetimibe, they showed a 32.57% increase and 19.69% decrease in ALT levels, respectively. The ALT assay (**Figure 3**) showed a steady increase in ALT levels in the first the three days when fed coconut oil medium, followed by a steady decrease of ALT levels in flies transferred out of the high fat medium into the control medium supplemented with ezetimibe.

In order to test for significance of the data collected, an independent t-test was used after the conclusion of the fifth

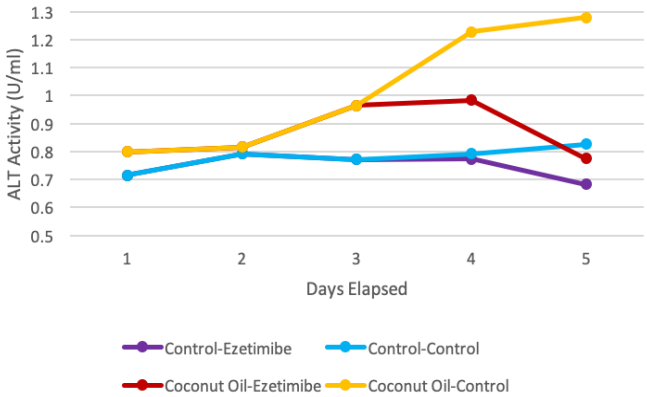

Figure 3. PALT levels measure over a period of five days. The average levels of ALT (U/mL in D. melanogaster were measured over five days. Five flies for each variable (Control-Ezetimibe, purple; Control-Control, blue; Coconut oil-Ezetimibe, red, and Coconut oil-control, yellow) were used for each measurement. Error bars represent a 95% confidence interval.

day. Comparing the flies given coconut oil medium who were transferred to either control or ezetimibe-containing medium, those given the drug treatment had significantly lower levels of triglycerides (p=0.011, **Figure 2**) and lower ALT levels than controls (p=0.00724, **Figure 3**). This statistic is important because it demonstrates that ezetimibe was able to lower both elevated markers of NAFLD. When comparing the weights of the flies kept in the control medium for three days to the flies kept in the coconut oil medium for the same time, there was no significant difference (p=0.0938, **Figure 4**). The test suggests that flies who had increases in triglyceride levels did not necessarily have an increase in weight. These results allowed us to reject the null hypothesis.

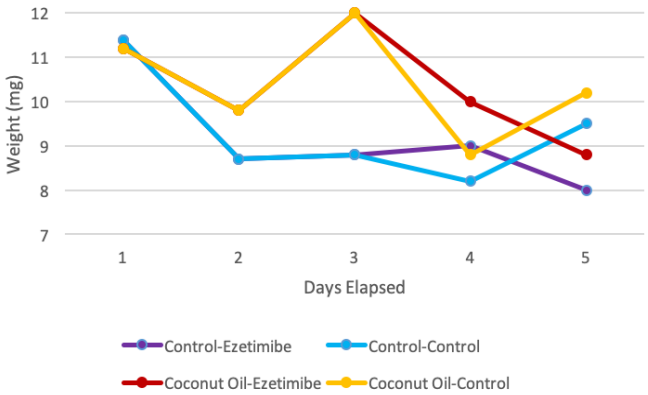

Figure 4. Average weight of *D. melanogaster.* The average weight (in milligrams) of the flies in each experimental medium condition over five days is depicted. Lines represent which medium the flies started in and were transferred to: Control-Ezetimibe (purple), Control-Control (blue), Coconut Oil-Ezetimibe (red), Coconut Oil-Control (yellow). Error bars represent a 95% confidence interval.

DISCUSSION

The goal of this scientific project was to test the hypothesis that ezetimibe could be a viable treatment of certain symptoms in a fly model of NAFLD. To test this hypothesis, flies were first given a high-fat diet to induce the disease and were then transferred to an ezetimibe- supplemented or control medium. Triglyceride, ALT, and weight measurements of the fruit flies were collected daily over a span of five days. The data collected showed that flies transferred from the high-fat diet to the control media supplemented with ezetimibe showed significant decreases in milligrams of triglycerides per mL and units of ALT after 48 hours. This supports the hypothesis that ezetimibe can lower triglyceride levels and ALT levels in flies previously treated with a high-fat diet. If ALT is a causative factor in disease, then returning ALT levels to normal with ezetimibe could prevent further liver damage. Triglyceride levels are one of the main pathologies of NAFLD because they represent the accumulation of fat as a result of the disease. Lowering triglycerides could also be a step toward preventing further liver damage and returning proper function to the liver. In order to further show ezetimibe's viability as an NAFLD treatment, more experiments on the drug's effect on other markers of NAFLD can be performed in flies. The promise of ezetimibe as a successful treatment for fruit flies could lead to a discussion on whether it could have the same effect in human patients with this disease.

During this study, while the assay procedures were followed as accurately as possible, there were some errors that may have occurred. Before being able to be assayed, the flies needed to be ground up in a tube using a glass rod. There was ample room for error during this process as grinding them uniformly each time proved to be difficult. Remnants of the flies would be left over on the rod frequently, and it is possible that some fly mass was lost in the process, which could affect measurements of triglycerides or ALT levels.

Regarding possible limitations of the experiment, a population of 140 flies was used for the duration of the experiment, but only 5 flies were assayed per data point. This decreased the power of the data as it is always better to have more flies assayed to support conclusions. Also, due to the lifespan of the flies in the high-fat diet and some outside factors, the experiment could only be run for five days when, perhaps, a longer experiment may have been more appropriate. Additionally, the amount of media each of the flies ate, if they ate it, was not examined and could be a possible source of error. Despite these possible faults, we still concluded that ezetimibe treatment lowered triglycerides and ALT levels, while further studies could address these possible caveats.

There were also limitations which may have affected the performance of this experiment. The experiment involved *D. melanogaster,* which is a biological organism. Using live organisms allowed for room for error as tests could only be performed postmortem. After death, natural biological processes may lead to the degradation of proteins and fats like the triglycerides tested. To try and avoid this, ground up flies were immediately placed in a buffer and were tested within 30 minutes of grinding. Also, the transferring method used was FlyNap, an anesthetic composed of trimethylamine, ethanol, and methanol, which puts flies to sleep for a couple of hours. Occasionally, flies would die and there is nothing we can do to prevent losing flies. In addition, the number of flies used in the experiment and assayed was limited because the flies used needed to be synchronized Flies needed to be the same age to limit the potential variation in levels of triglyceride depending on their maturation. Also, younger flies could have increased resistance to the physiology of NAFLD due to their increased metabolic activity compared to the older flies. Observing the effect of ezetimibe over a greater period of time would allow for more calculated conclusions on the effectiveness of the drug. For example, it could show if ezetimibe decreased in effectiveness over time and perhaps if transferring flies to just the control medium could prove to be more effective long term. Furthermore, the number of flies used per assay was limited to the two variables of the experiment and the two assays that needed to be run. This experiment repeated with

a larger number of flies tested per group would strengthen conclusions made from this data.

The data and observations collected in this experiment on *D. melanogaster* showed the promise of ezetimibe as a future medication for patients with NAFLD. In the future, more extensive clinical trials can be performed with NAFLD patients to show whether this drug could have the same effect in humans. More variables could be used including a placebo group in a bigger study run on a large number of patients. Additionally, more tests can be run to observe how this drug affects the disease as well as other effects it may have on the human body besides fats and liver disease. If clinical trials go well, ezetimibe could become an approved treatment for patients with NAFLD.

METHODS

Control Medium Preparation

To make the control diet, we used medium from a 1 kg bag of Formula 4-24® Instant *Drosophila* Medium (please provide the name of the manufacturer here). Enough medium was measured to fill a third of the vial where the *D. melanogaster* flies were being kept. 10-15 mL of water was added to each vial, and the medium was mixed completely.

High-Fat Medium Preparation

Using the same 1 kg bag of Formula 4-24® Instant *Drosophila* Medium (again manufacturer name should be added), medium was measured to fill a third of seven more vials. Coconut oil was added to make up about 30% of the medium. 8 g of Instant *Drosophila* Medium was mixed with 6 mL of water in a vial. Then, 6 mL of coconut oil was added to make up 30%, and mixed with the other contents of the medium.

Preparation of Control Medium Supplemented with Ezetimibe

Control medium was prepared as described above. The ezetimibe supplement was prepared by using a mortar and pestle to grind one 10 mg tablet of ezetimibe. Then 2 mg of ezetimibe was diluted in one mLr of water. Separately, 75 mL of water was prepared. 5.6 uL were micro-pipetted out from the 75 mL of water. 5.6 uL of the 2 mg/mL ezetimibe solution was then micro-pipetted into the 75 mL of water. Then, 10 mL of ezetimibe solution was added to 10 g of control medium in four vials, which would give a final ezetimibe dose of approximately 1.49×10^{-7} mg/ml medium, the normal dosage of ezetimibe for a human patient adjusted to the average body weight of a fly.

The vials were labeled by which diet they contained. Using FlyNap®, flies were transferred from fly culture tubes purchased from Carolina Biological into the newly made tubes. Ten flies were transferred into each of the 14 diet vials, 7 control tubes and 7 coconut oil medium tubes, for a total of 140 flies being transferred. After the flies were transferred, all 14 vials were plugged to ensure no flies were lost. The number of flies in each vial was recounted to ensure each vial had the same number of flies.

Weighing the Flies: Before the flies were weighed, FlyNap® was used to temporarily sedate the flies so they could be taken out the vial without escaping. While the flies were still asleep, they were weighed on an ultra-sensitive scale in pairs of five prior to being assayed. The ten flies in each vial were split into two groups of five for the two assays.

ALT Activity Assay Kit

Using the ALT Assay Kit purchased from Cayman Chemicals, the kit reagents and assay were prepared and run according to manufacturer's recommendations. Five flies from each vial were ground in a test tube with 5-10 mL of cold buffer and centrifuged in a 15 mL centrifuge tube for fifteen minutes. In a 96-well plate, positive control wells were made using the kit and the fly samples were added to sample wells using instructions from the kit. Then, the plate was run through a microplate reader, and data was collected on the ALT levels in the flies.

Triglyceride Colorimetric Assay Kit

Using the Triglyceride Assay Kit, the kit reagents and assay were prepared and run according to manufacturer's recommendations. Five flies from each vial were ground in a test tube with a glass rod in 2 mL of NP-40 Substitute Assay Reagent and centrifuged for ten minutes. Samples were placed in a well plate with the addition of Triglyceride Standard under the instructions of the kit, and were then run through a microplate reader. Data was then collected on the triglyceride levels in flies.

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