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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

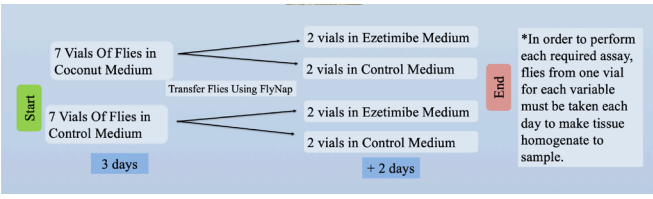

Figure 1. Procedure flow chart. Flies were either kept in coconut oil or control medium for a three-day period and were then transferred to either an ezetimibe or control medium for a two-day period, creating four variables. Flies from each variable were assayed over the course of the five-day period.

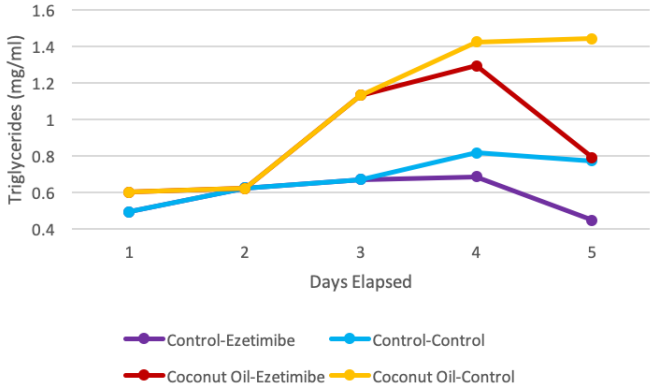

Figure 2. Triglyceride levels measured over a period of five days. The average levels of triglycerides (mg/mL) in D. melanogaster were measured over five days. Five flies for each variable (Control-Ezetimibe, purple; Control-Control, blue; Coconut oil-Ezetimibe, red, and Coconut oil-control, yellow)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

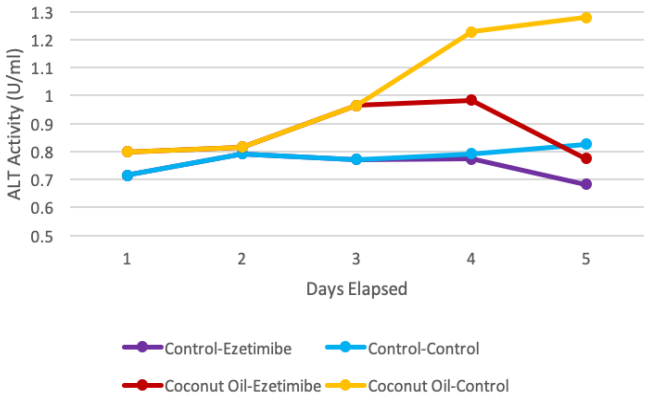

Figure 3. PALT levels measure over a period of five days. The average levels of ALT (U/mL in D. melanogaster were measured over five days. Five flies for each variable (Control-Ezetimibe, purple; Control-Control, blue; Coconut oil-Ezetimibe, red, and Coconut oil-control, yellow) were used for each measurement.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

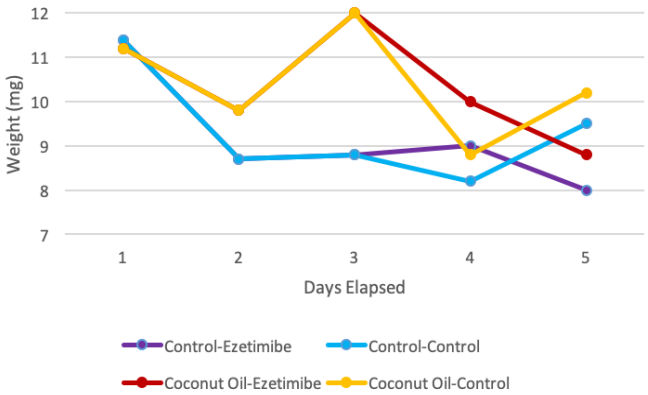

Figure 4. Average weight of *D. melanogaster.* The average weight (in milligrams) of the flies in each experimental medium condition over five days is depicted. Lines represent which medium the flies started in and were transferred to: Control-Ezetimibe (purple), Control-Control (blue), Coconut Oil-Ezetimibe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

1. Hengstler, Jan G., and Leif Dehmelt. "Metabolic Alterations in Non-Alcoholic Fatty Liver Disease (NAFLD): Consequences of AGXT Downregulation on Glyoxylate Detoxification." *Chemische Biologie*, 2017, https://eldorado.tu-dortmund.de/bitstream/2003/36084/1/Dissertation_Gianmoena.pdf.
2. Bhardwaj, A. "Cholesterol Therapy Thats Not Chopped Liver." *Science Translational Medicine*, vol. 6, no. 262, Dec. 2014, doi:10.1126/scitranslmed.aaa2057.
3. Paschos, P, and K Paletas. "Non Alcoholic Fatty Liver Disease and Metabolic Syndrome." *Hippokratia*, vol. 1, 13 Mar. 2009, pp. 9–19., https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2633261/.
4. Manco, Melania. "Insulin Resistance and NAFLD: A Dangerous Liaison beyond the Genetics." *Children*, vol. 4, no. 8, 2017, p. 74., doi:10.3390/children4080074.
5. Fabbrini, Elisa, *et al*. "Obesity and Nonalcoholic Fatty Liver Disease: Biochemical, Metabolic, and Clinical

Implications." *Hepatology*, vol. 51, no. 2, Sept. 2009, pp. 679–689., doi:10.1002/hep.23280.
6. Schindhelm, Roger K., *et al*. "Alanine Aminotransferase as a Marker of Non-Alcoholic Fatty Liver Disease in Relation to Type 2 Diabetes Mellitus and Cardiovascular Disease." *Diabetes/Metabolism Research and Reviews*, vol. 22, no. 6, 2006, pp. 437–443., doi:10.1002/dmrr.666.
7. Clader, J.w. "Ezetimibe." *Comprehensive Medicinal Chemistry II*, 2007, pp. 65–82., doi:10.1016/b0-08-045044-x/00291-1.
8. Diop, Soda Balla, *et al*. "High Fat Diet Feeding and High Throughput Triacylglyceride Assay in *Drosophila Melanogaster*." *Journal of Visualized Experiments*, no. 127, 2017, doi:10.3791/56029.