

Observing effects of resolving leaky gut on sugar, fat, and insulin levels during type 1 diabetes in fruit flies

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SUMMARY

The goal of this project was to analyze one of the causes of type 1 diabetes (T1D), increased intestinal permeability. The microbiota imbalance caused by gut leakiness leads to an autoimmune cascade causing beta cell destruction; the focus of this project was restoring the integrity of the intestinal lining, thus reducing symptoms of T1D. As such, a hypothesis was formed that if solutions of sodium butyrate and casein are administered to *Drosophila* that display symptoms of T1D, then they will experience decreased symptoms including lower trehalose and triglyceride levels and higher *Drosophila* insulin-like peptides (DILP) levels over time. After attempting to resolve impaired intestinal barrier function, the changes caused by T1D, specifically trehalose, triglyceride, and *Drosophila* insulin-like peptide levels, were examined. After testing for intestinal permeability, wild type and T1D mutant flies were administered with diets of sodium butyrate and hydrolyzed casein, both of which strengthen intestinal tight junctions, along with a normal diet to act as a control group. Testing for symptoms of T1D was done over a period of 10 days for each group in order to measure the restoration of function and effectiveness of the treatment. The results indicated that intestinal permeability was resolved through the use of both chemicals and that symptoms of T1D generally decreased. Triglyceride and trehalose levels decreased, while DILP levels remained consistent, indicating the inhibition of beta cell destruction. *Drosophila* has been shown to work well as a model organism for T1D, so there is a strong reason to look into the effectiveness of applying the same treatment for T1D in mammals.

INTRODUCTION

Diabetes is a disease in which the body cannot regulate blood sugar. Blood sugar is regulated by two hormones, glucagon and insulin, which increase and decrease glucose levels, respectively. T1D makes up approximately 10% of all diabetes cases. In T1D, an autoimmune disease causes the destruction of insulin-producing beta cells in the pancreas and is typically of genetic origin (1).

Insulin secreted by beta cells is imported by the sugar transporters GLUT1 and GLUT2. It binds to insulin receptors in the cell membrane, causing a phosphorylation cascade of the insulin receptor substrate (IRS), PI3 kinase, and AKT (or protein kinase B in *Drosophila*) (2). The affected AKT

phosphorylates the FOXO transcription factor, which is responsible for the expression of genes necessary for the stress response and survival (3). Therefore, inhibition of insulin signaling components in this way leads to elimination of activation of FOXO and decreased blood sugar regulation.

Based on the idea of using *Drosophila* as a model, the functional and histological aspects of the small intestine and human T1D have been investigated, though these attempts have been limited in number. Regarding intestinal permeability, the clearance of lactulose and mannitol in patients with T1D has been investigated with different results. These results have led some to believe that this loss is an important characteristic of T1D and that poor regulation of the zonulin protein due to the effects of this loss may result in the occurrence of the disease (4).

Class I PI3 kinases consist of a regulatory subunit and a catalytic subunit, each of which exists in different isoforms. Recruitment and activation of PI3K depend on the binding of the two SH2 domains in regulatory subunits with IRS-made phosphorylated by tyrosine. This leads to activation of the catalytic subunit, which phosphorylates phosphatidylinositol-4, 5-bisphosphate (PIP2) rapidly to produce the second lipid messenger of phosphatidylinositol (3,4,5) triphosphate (PIP3). The latter recruits AKT into the plasma membrane, where phosphorylation activates it and induces a subsequent signal transfer (5).

The receptor DlnR or InR has been reported to act in the same way as the mammalian insulin receptor in the sense that it has tyrosine kinase activity and causes auto-phosphorylation in response to human insulin. These studies and many others have made it clear that insulin and insulin-like routes are shared between flies and humans. Interestingly, insects express a large number of DILPs, from eight in *Drosophila* to many others in other invertebrates, but only one insulin-like receptor. Different DILPs probably play many roles in animals, and the same metabolic functions seem at least partially unnecessary (6). Significantly, insects appear to have many ligands for a receptor, while mammals have receptors with partially redundant functions, but a limited number of ligands. The cause of this discrepancy between insects and mammalian lines remains an unsolved problem in this area, but the importance of their similarity cannot be understated.

Sodium butyrate is the sodium salt of butyric acid, which causes inhibition of HDAC, promoting histone deacetylation. Some studies have been conducted which shows that on the onset of T1D, the intestines have a defect which causes increased permeability. This intestinal permeability causes inflammation which leads to pancreatic beta cell destruction later on. This happens through a microbiota imbalance that

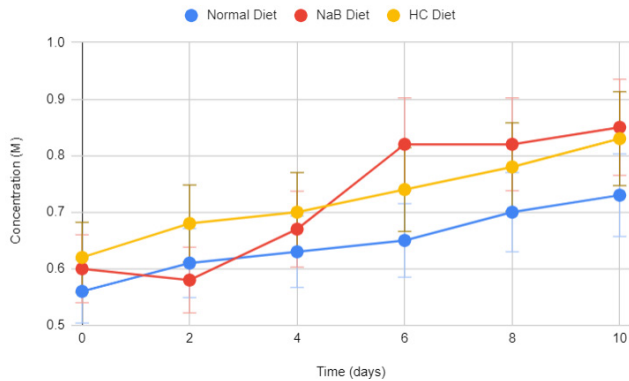


Figure 1: Effect of different diets on triglyceride levels in wild-type flies. Concentration of triglyceride levels was monitored over the course of 10 days. Error bars represent standard deviation. All three diets resulted in a generally linear increase in triglyceride levels due to further growth, development, and food consumption of the *Drosophila*. Both sodium butyrate (NaB) and hydrolyzed casein (HC) resulted in slightly higher levels than the normal diet.

exacerbates gut leakiness and causes an autoimmune cascade, leading to beta cell autoimmunity and insulinitis (6). Butyric acid causes the down-regulation of zonulin, strengthening the tight junctions and decreasing permeability, with hydrolyzed casein having a similar purpose. Based on this information, it appeared reasonable to hypothesize that if solutions of sodium butyrate and casein are administered to *Drosophila* that display symptoms of T1D, then they will experience decreased symptoms including lower trehalose and triglyceride levels and higher DILP levels over time. (7).

RESULTS

In groups of wild type flies given a normal diet, sodium butyrate diet, and hydrolyzed casein, levels of trehalose and triglycerides generally increased linearly. This is due to the fact that over time, the flies experienced greater growth and development, causing an increase in metabolism. Glucose levels were initially gathered with the Glucose HK assay, and a standard curve was used to convert to trehalose levels due

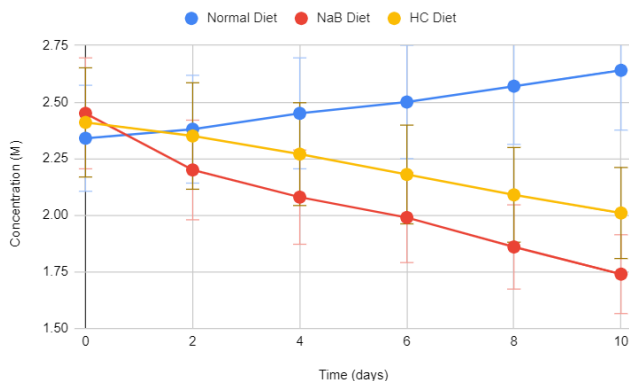


Figure 3: Effect of different diets on triglyceride levels in HNF4 flies. Concentration of triglyceride levels was monitored over the course of 10 days. Error bars represent standard deviation. Normal diet resulted in a linear increase in triglyceride levels as diabetic symptoms slowly worsened due to lack of treatment. Both sodium butyrate (NaB) and hydrolyzed casein (HC) resulted in far lower triglyceride levels than the normal diet, with NaB having a greater effect. Shows the effectiveness of the treatment.

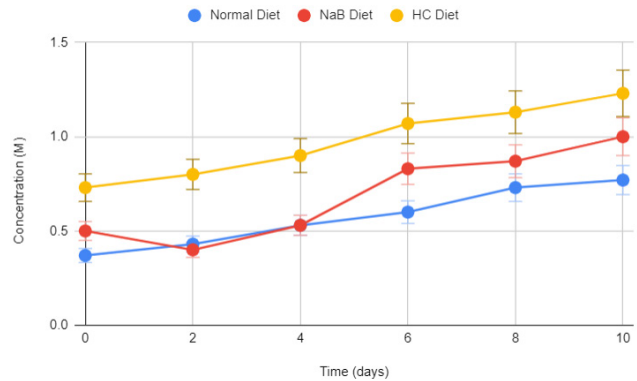


Figure 2: Effect of different diets on trehalose levels in wild-type flies. Concentration of trehalose levels was monitored over the course of 10 days. Error bars represent standard deviation. All three diets resulted in a generally linear increase in triglyceride levels due to further growth, development, and food consumption of the *Drosophila*. Both sodium butyrate (NaB) and hydrolyzed casein (HC) resulted in slightly higher levels than the normal diet.

to being the primary circulating sugar. Data was not entirely consistent due to the fact that the flies may have eaten different amounts of food, but as the data still increased over time it is clear that the trend remained consistent. In terms of mutant flies, administration of both chemicals displayed a clear decline in type 1 diabetic symptoms, giving enough information to support the hypothesis. The data shows that when wild-type and HNF4 mutant flies were given a normal diet, their trehalose and triglyceride levels generally increased, with the mutants experiencing a much greater increase (Figures 1-2). The same held true for the wild-type flies when given the diets of both chemicals; however, the mutant flies experienced a decrease in both levels, indicating that T1D affected the flies as expected (Figures 3-4).

When both chemicals were administered, the PI3K92E assay indicated increases in DILP levels, illustrating the same corresponding change (Figures 5-6). Whether the wild type flies were given sodium butyrate or hydrolyzed casein did not significantly impact the trend of the data, but the sodium butyrate diet generally had a greater effect

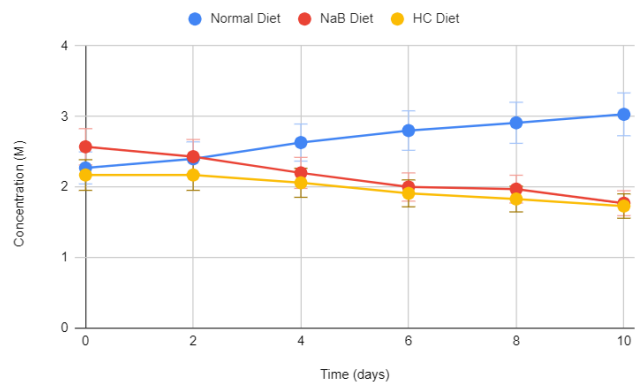


Figure 4: Effect of different diets on trehalose levels in HNF4 flies. Concentration of trehalose levels was monitored over the course of 10 days. Error bars represent standard deviation. Normal diet resulted in a linear increase in trehalose levels as diabetic symptoms slowly worsened due to lack of treatment. Both sodium butyrate (NaB) and hydrolyzed casein (HC) resulted in far lower trehalose levels than the normal diet. Shows the effectiveness of the treatment.

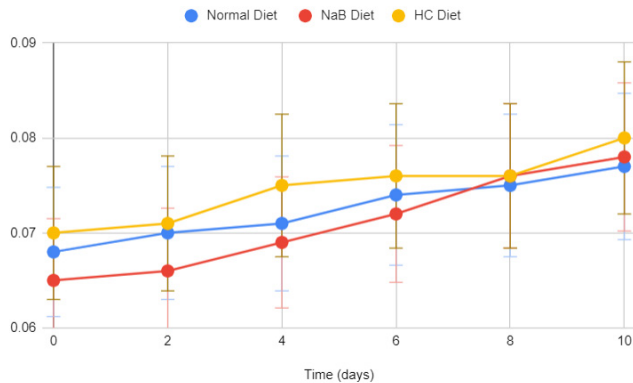


Figure 5: Effect of different diets on DILP levels in wild-type flies. Concentration of DILP levels was monitored over the course of 10 days. Error bars represent standard deviation. All three diets resulted in a generally linear increase in DILP levels due to further growth, development, and food consumption of the *Drosophila*.

on reducing symptoms than hydrolyzed casein, due to its increased absorption in the intestines. As illustrated by the data in the graphs, triglyceride and trehalose levels declined, while insulin levels remained mostly constant, illustrating that the damage caused by intestinal permeability was reversible and beta cell destruction was slowed.

The Smurf assay was used to analyze the degree of the intestinal permeability and was administered to all flies. As illustrated by the data gathered, the Smurf assay showed that prior to administration of these chemicals, the *Drosophila* with the HNF4 mutation were bluer and had more severe permeability, as expected (Figure 7). An ANOVA test was done on all six groups to analyze the data obtained. For triglyceride and trehalose levels, the F statistic was greater than the F crit value and the p value was less than 0.05, indicating that there was variance between groups and the data was statistically significant (Table 1). However, neither of these applied to PI3K92E levels, illustrating their consistency and the likelihood that their slight variation was more random.

DISCUSSION

The main limitation of this experiment was the fact that the colorimetric assay for trehalose quantification was the most

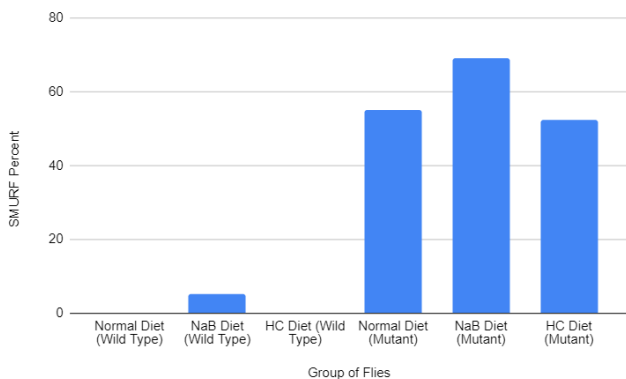


Figure 7: Smurf assay for prevalence of intestinal permeability. The percentage of flies that were deemed to be Smurfs (blue color) was monitored for all six groups tested. Wild-type groups had almost no *Drosophila* with increased intestinal permeability, while the majority of mutant flies experienced leaky gut syndrome. The Smurf assay also indicated which mutant flies would be treated.

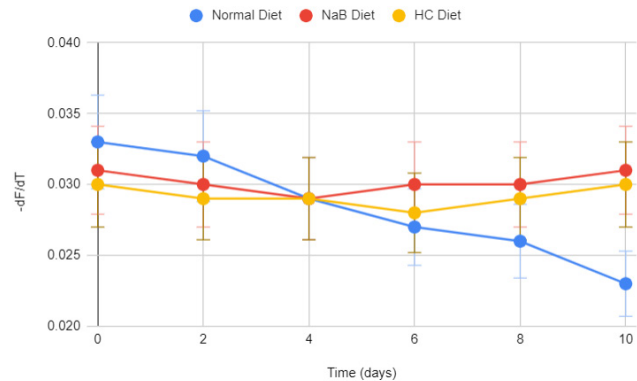


Figure 6: Effect of different diets on DILP levels in HNF4 flies. Concentration of triglyceride levels was monitored over the course of 10 days. Error bars represent standard deviation. Normal diet resulted in a decrease in DILP levels as diabetic symptoms worsened due to a lack of treatment. Both sodium butyrate (NaB) and hydrolyzed casein (HC) resulted in generally stable DILP levels compared to the normal diet, with NaB having a slightly greater effect. Shows the effectiveness of the treatment.

viable method, but the quantities determined are likely higher than general despite the glucose to trehalose conversion. This did not affect the trend at all, and the actual quantities are still mostly accurate, but it is a limitation worth noting. Analyzing the actual immune pathway and the change that resulted was not feasible, meaning that a certain aspect of the leaky gut pathogenic factor was not accounted for, partially limiting its usefulness. Additionally, although the Smurf assay identified flies with intestinal permeability, the only way to illustrate the function of the treatment was the decline in blue appearance over time, but this was difficult to verify in some cases. Alternatively, the fact that at certain intervals, the levels recorded did not change much, illustrating that the effectiveness of this treatment was not entirely consistent. The spectrophotometer likely was not entirely consistent at all times, especially the blank reagents, which was another source of possible error.

The results of the experiment have illustrated that the beta cell destruction caused by the increased intestinal permeability can be slowed by reversing the fact of leaky gut. After analyzing the similarities between the *Drosophila* and mammalian insulin pathway, the effectiveness of this treatment in reducing diabetic symptoms can be analyzed in humans. These findings illustrate the strong possibility of finding additional types of treatment due to a stronger link

Table 1: One-way ANOVA Results. F statistic of mutant triglyceride levels and both trehalose levels are greater than F critical value of 3.098391224. P-value of mutant triglyceride levels and both trehalose levels are both less than 0.05. Statistical significance and variance between groups is shown with these three groups. Neither of these applied to wild-type triglyceride levels and both PI3K92E levels. Sum of Square (SS) and Mean Square (MS) of these latter three groups are lower, also indicating less variance.

Category	SS	MS	F	P-value
Wild-type Triglyceride Levels	0.02403333333	0.01201666667	1.499376126	0.2548980119
Wild-type Trehalose Levels	0.5215444444	0.2607722222	6.351003951	0.01004269046
Wild-type PI3K92E Levels	0.0004077777778	0.0002038888889	1.154814349	0.3416066388
Mutant Triglyceride Levels	0.3362111111	0.1681055556	6.326628753	0.010117623692
Mutant Trehalose Levels	1.563544444	0.7817722222	11.02796194	0.001133057892
Mutant PI3K92E Levels	0.00001011111111	0.00000505555556	0.9848484848	0.3963955674

between T1D and increased intestinal permeability being illustrated. Inspection into additional types of solutions to see which types of treatments are most effective should be another factor that could be looked into. The specific dose of a 0.1 M solution was chosen as previous studies investigating other diseases such as Parkinson's have determined that this concentration is adequate to be effective while also not causing harm to the *Drosophila* in any way (8). Additional concentrations of the solutions should be looked into as the speed of the restoration of intestinal integrity could differ from what is expected in relation to concentration. Other types of mutant flies that display T1D symptoms are available, and as such, whether the effects of them are different could be analyzed. Continuing from this, despite *Drosophila*'s effectiveness as a model organism, other model organisms could be investigated as the way that the insulin system functions in relation to the intestinal tract could be different (9).

MATERIALS AND METHODS

Flies were separated into six groups, three of which are wild type *Drosophila* (Bloomington #25210) and three of which have an HNF4 knockout mutation (Bloomington #44398). The Smurf assay was used on each group to determine which flies displayed increased intestinal permeability and which flies should be targeted with the treatment (10). Afterwards, sodium butyrate and hydrolyzed casein solutions were both administered to two groups of wild type flies and two groups of mutant flies, both at 0.1 M. The trehalose quantification method was used on one group of *Drosophila* by homogenizing them in Tris buffer and trehalase stock (made from porcine trehalase) to digest the trehalase into glucose and using the glucose HK assay (11). Specifically, solutions of the test sample, the assay reagent, and water would be combined and read at 340 nm against a reagent blank. The lipid assay was performed by homogenizing *Drosophila* in a microcentrifuge tube, adding 0.5 mL of the 1:1 chloroform-methanol solution, and 0.2 mL of 1 M sulfuric acid, then heating it for 10 minutes. Afterwards, 4.3 mL of vanillin-phosphoric acid reagent was added and the solution was read against a reagent blank at 525 nm (12,13). Both assays utilized a spectrophotometer to test for absorbance at various wavelengths to convert to concentration. The PI3K92E assay was performed by RNA extraction using TRI reagent, conversion to cDNA using an RNA to cDNA kit, and qPCR analysis with the MIC PCR machine to determine DILP levels.

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