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The Effect of Alpha Lipoic Acid on Cardiac Autonomic Neuropathy in Danio rerio

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I. Abstract

Even though poor glycemic control puts diabetics at risk for a variety of disease complications, there are many barriers to achieving adequate blood glucose regulation. Diabetic cardiac autonomic neuropathy (CAN) is a progressive autonomic denervation of the cardiovascular system resulting from chronic hyperglycemia and characterized by orthostatic hypertension, sinus tachycardia, low heart rate variability, and fatal arrhythmias. Available CAN therapeutics are limited to symptom alleviation, and do not prevent or reverse cardiac nerve damage. Limited evidence from clinical trials suggests the antioxidant Alpha Lipoid Acid (ALA) prevents CAN progression (Lee et al., 2017; Serhiyenko et al., 2020; Tankova, Koev & Dakovska, 2004; Ziegler et al., 1997). This study tested the effect of ALA in the preclinical setting using a zebrafish model with diet induced hyperglycemia. Zebrafish were divided into four groups: balanced diet, high carbohydrate diet, balanced diet with ALA tank water, or high carbohydrate diet with ALA tank water. Noninvasive techniques were used to quantify symptoms of hyperglycemia and cardiac autonomic neuropathy. Hyperglycemic behaviors were analyzed using novel tank diving tests while light cardiography was done to measure heart rate and heart rate variability. The results indicate that a six-week high carbohydrate diet in zebrafish inhibits weight gain and promotes bradycardia under anesthesia. Additionally, ALA treatment may reduce anxious behavior during novel tank diving.

II. Introduction

Cardiac autonomic neuropathy (CAN) is the progressive degeneration of neurons innervating the cardiovascular system. CAN develops in about 20% of type one and type two diabetics (Spallone et al., 2011). Chronic hyperglycemia causes oxidative stress. Under hyperglycemic conditions, glucose metabolism produces excessive neurotoxic reactive oxygen species (ROS). In CAN, the degeneration of the parasympathetic vagus nerve creates an autonomic imbalance. The disproportional sympathetic control manifests as the hallmark symptoms of CAN: tachycardia, decreased heart rate variability, and orthostatic hypotension (Dimitropoulos et al., 2014). Additionally, CAN often causes fatal arrhythmias increasing the mortality rate of diabetics by two to three times (Balcıoğlu & Serhat, 2015).

Strict glycemic control can decrease the likelihood of CAN development, but this is not feasible for all diabetics. Current treatments are limited to symptom alleviation and do not prevent or reverse nerve damage (Pop-Busui et al., 2017). Cardiac autonomic neuropathy's full pathogenesis is still relatively unknown limiting the therapeutic targets available (Dimitropoulos et al., 2014). Limited clinical studies have suggested the over-the-counter antioxidant, alpha lipoic acid (ALA), may prevent cardiac neurodegeneration, but the results are conflicting. ALA promotes increased cellular uptake of glucose and reduces ROS, so it is hypothesized to prevent CAN development by preventing oxidative degeneration of neurons (Lee et al., 2017; Serhiyenko et al., 2020; Tankova, Koev & Dakovska, 2004; Ziegler et al., 1997).

Preclinical research is needed to further elucidate CAN's mechanism and how it might be altered by ALA. Preclinical models of CAN have been established in mice, rats, pigs and rabbits, but ALA treatment has not been tested in these models (Stables, Glasser, & Feldman, 2013). Zebrafish have not been used previously to study diabetic cardiac autonomic neuropathy. The

zebrafish heart and intracardiac nervous system are functionally similar to the mammalian heart making them a useful model for cardiac autonomic neuropathy research (Beffagna, 2019; Stoyek et al., 2016). Based on the cardiovascular similarity of the zebrafish heart to humans we aimed to develop a model of diabetic CAN in zebrafish with translational value. Using noninvasive procedures to induce and measure CAN in this model permits a long-term study to be done to track disease progression. A CAN model with face and predictive validity is crucial to better understand the disease and develop novel treatment options.

A pilot study was done to test if a high carbohydrate diet can induce diabetic CAN in zebrafish and if hyperglycemic state, heart rate and heart rate variability can be accurately and noninvasively measured. Establishing non-invasive measurements of CAN is needed to limit unnecessary stress that can alter test results and to prevent premature death permitting more time to study disease progression. Zebrafish ECGs and BGL tests are invasive but well-established tests. Light cardiography and novel tank diving are non-invasive tests that are not well established. The ECGs and blood glucose test were used as a reference to support light cardiography and novel tank diving test results for the main study.

ALA's Influence on heart rate, heart rate variability, and hyperglycemic state was non-invasively tested in a diet-induced CAN zebrafish model.

III. Methods

All methods were approved by The University of Akron Institutional Animal Care and Use Committee (IACUC) according to protocol numbers 22-02-22 BFD and 22-11-13 BFD.

A. Pilot Animals

Twenty-eight adult zebrafish were split into two groups of 14 each housed in 2 liters of water between 27℃ ∓ 2. Both groups were fed 5% of their body weight once a day. One group had high carbohydrate diet of 25% Gemma Micro 300 and 75% freeze-dried pineapple while the other receive a balanced diet of only Gemma Micro 300 (Skretting, Tooele, UT). For two months, the fish received their assigned diet and underwent weekly light cardiography and novel tank diving tests. During the first and last trial of testing, an ECG was done immediately after light cardiography while the fish were still anesthetized. During the last trial of testing, four high carbohydrate fish and four control fish were euthanized to measure their blood glucose level (BGL).

B. Main Project Animals

Sixty adult zebrafish were split into four groups of 15: balanced diet, high carbohydrate diet, balanced diet with alpha lipoic acid treatment, and high carbohydrate diet with alpha lipoic acid treatment. Each group was housed in 8 liters of water between 27℃ ± 2. Zebrafish were fed 5% of their body weight once daily during their 14-hour light cycle. The balanced diet was composed of Gemma Micro 300. The high carbohydrate diet was 25% Gemma Micro 300 and 75% freeze-dried pineapple. ALA was administered in tank water at 0.002%. A dose of about 0.002% ALA was used for safe aquatic respiration and a potential therapeutic effect based on previous literature (Camiolo et al. 2019; Carota 2022). Bi-weekly light cardiography and novel tank diving tests were done for 6 weeks.

C. Novel Tank Diving

Following the protocol by Wang et al. (2020), zebrafish were added to an empty 10-gallon tank and their behavior was recorded for five minutes. Tape on the outside of the tank was used to divide the top and bottom halves. Time spent in the top half of the tank and entries made into the top half of the tank from the bottom half was documented for each test. The novel tank was used to quantify hyperglycemic behaviors. Less time spent in, and entries made into the top half of a new tank can be indicative of anxiety caused by hyperglycemia (Wang et al., 2020).

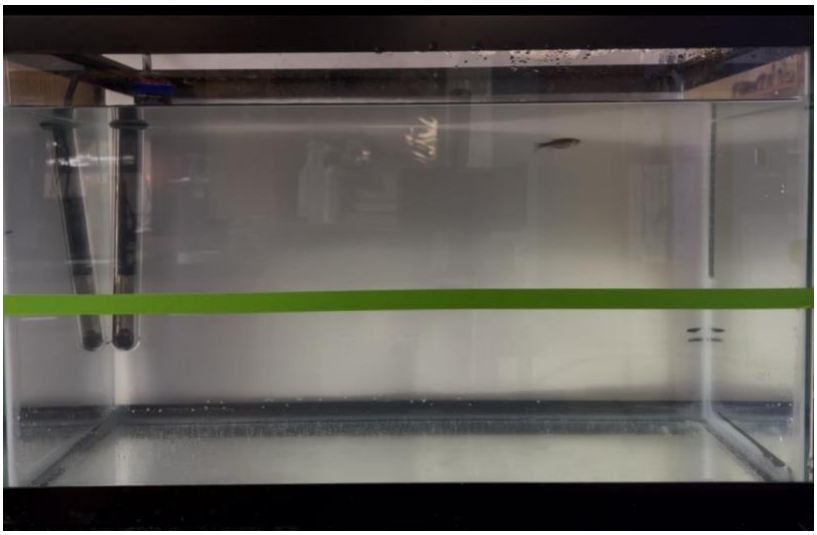

Figure 1. Novel tank diving tests were done in a 20” x 12” x 10” 10-gallon tank. For testing the tank was filled with 8 gallons. Tape was placed at the 4-gallon mark to separate the top and bottom half of the tank.

D. Blood Glucose Test

During the pilot study, and immediately after their final novel tank diving trial, eight zebrafish were euthanized with an overdose of 0.05% MS-222 buffered with sodium bicarbonate to a pH of 7.0. After confirmation of death, fish were decapitated at the pectoral girdle and a

blood glucose test strip was placed directly on the exposed dorsal artery to collect blood (Eames et al., 2010, Marks et al., 2012). Following the manufacturer's instructions, a Nova Max glucometer was used to read the BGL of the blood retrieved from the test strip.

E. Anesthesia and Recovery

Before each light cardiography and ECG trial, zebrafish were anesthetized with 0.01% tricaine methanesulfonate (MS-222) buffered to a pH of 7.0 with equal parts sodium bicarbonate. Anesthetized zebrafish were placed into the slit of a damp sponge with the ventral surface of the fish exposed. Once testing was complete, zebrafish were transferred to a recovery tank with an air stone and monitored for successful recovery.

F. Light Cardiography

Light cardiography measured heart rate and heart rate variability. The ventral surface of an anesthetized zebrafish was illuminated and the oscillation in brightness over the heart was used to calculate heart rate and heart rate variability (Mousavi & Patol, 2020). The light cardiography technique was based on the procedure by Mousavi & Patol (2020). On the ventral side of an anesthetized zebrafish, the skin over the atrium and ventricle was illuminated with a cold light source. This image was observed under a stereoscope and recorded with a camera for one minute. The change in pixel intensity, brightness of the pixel on the ventral surface over the heart, was recorded. The difference in the brightness of the ventral surface of the fish over time was used to determine the diastolic and systolic periods. The frequency of change in pixel intensity and variation between changes in pixel intensity was used to measure heart rate and heart rate variability respectively.

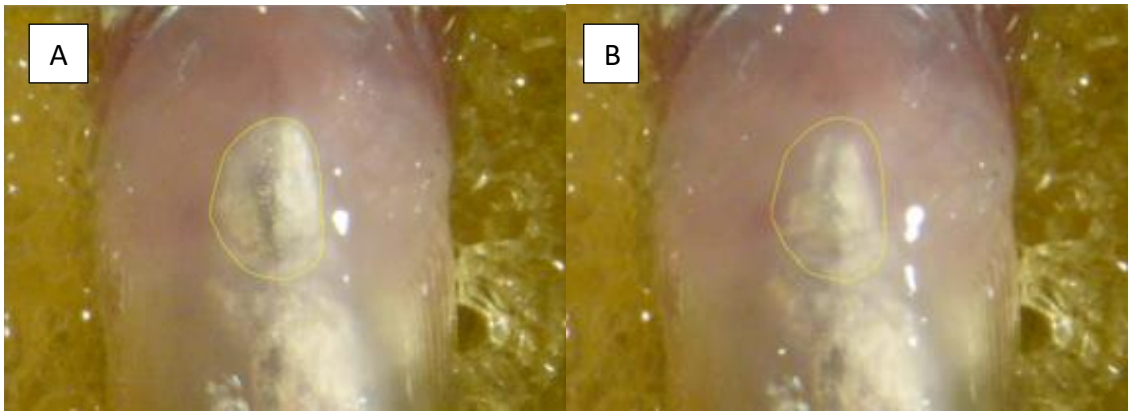

Figure 2. Ventral surface of an anesthetized zebrafish in the slit of a sponge under a microscope during light cardiography. The average pixel intensity inside the yellow line outlining the ventricle is measured every 1/30 of a second for 60 seconds. Oscillations in pixel intensity correspond with periods of systole and diastole. (A) Pixel intensity peaks during ventricular relaxation (B) Pixel intensity decreases during ventricular contraction.

G. Electrocardiogram (ECG)

The ECG protocol done during the pilot trial was based on a procedure by Zhao et al. (2019). Three needle electrodes were placed about 1 mm deep into the ventral surface of anesthetized zebrafish musculature to form a bipolar lead. The positive electrode was placed near the midline about one mm above the level of the operculum. The negative electrode was placed about one mm left of the positive electrode and below the base of the ventricle. The reference electrode was placed near the anal region. The ECG was recorded and analyzed using LabPro software.

Figure 3. An anesthetized zebrafish in the slit of a sponge during an electrocardiogram. A bipolar lead created with three electrodes: a positive electrode placed above the operculum at the midline, the negative electrode placed below apex of the ventricle, and a reference electrode placed at the anal region.

H. Statistics

Each novel tank diving trial was recorded, and videos were reviewed to manually count entries made into the top and record time spent in the top half. The blood glucose concentration was read from the glucometer and documented.

Light cardiography was recorded for one minute. Adobe Premiere was used to compress the video to 30 frames per second, then a tiff stack was created comprised of 1800 images, one per frame in the video. Using ImageJ, the ventricle was outlined and the average pixel density in the region was measured for each image in the stack. A peak in pixel density was marked at each timepoint that had a pixel density greater than the previous three images and the following three images. The number of peaks in 60 seconds was used to determine heart rate. The standard

deviation of the time between peaks was used to determine heart rate variability. Electrocardiography was also performed for one minute. The LabPro software recorded heart rate and heart rate variability.

An ANOVA was performed for each experiment to determine if there was significant difference among groups ($p<0.05$). Tukey post-hoc analyses were done for tests that had a significant difference between groups. A Pearson correlation test was done to determine if blood glucose levels taken during the pilot study corresponded with time spent in and entries made into the top half of the novel tank.

IV. Results

Zebrafish underwent light cardiography to measure heart rate and heart rate variability and novel tank diving to test for behaviors consistent with hyperglycemia. The pilot showed that these tests are repeatable due to both tests having 100% survival rate and no overt signs of distress. Electrocardiograms, however, were more invasive and resulted in an overall death rate of 44% due to the nicking of the heart either during insertion or removal of electrodes, and thus, subsequent exsanguination while under anesthesia.

At week 0 of the pilot study, light cardiography measurements resulted in a significantly higher heart rate than electrocardiography ($p < 0.001$). At week 8, light cardiography measurements resulted in a significantly higher heart rate than electrocardiography in balanced diet zebrafish only ($p= 0.018$). During the pilot, a high carbohydrate diet did not cause a significant difference in heart rate during electrocardiography or light cardiography. The high carbohydrate diet did not have a significant effect on novel tank diving behavior or blood glucose concentration. Despite not differing between groups, a Pearson correlation showed

positive correlation between blood glucose concentration and both time spent in the top half of the tank (r= 0.908) and entries made into the top half of the novel tank (r= 0.699).

In the main study, zebrafish fed a balanced diet had a significant weight gain after 6 weeks of their diet. The weight gain was significant in balanced diet zebrafish both treated with ALA ($p< 0.001$) and those not treated with ALA ($p= 0.0042$). At week 4, among zebrafish that were given a high carbohydrate diet, the fish also treated with ALA spent significantly more time in the top half of the novel tank ($p= 0.0339$). This trend did not continue at week 6. After 6 weeks among zebrafish fed a balanced diet, those treated with ALA spent more time in the top half of the novel tank ($p= 0.0137$). Heart rates of high carbohydrate diet zebrafish with ALA treatment ($p=0.0114$) and without ALA treatment ($p= 0.0202$) were significantly lower than zebrafish with a balanced diet and ALA treatment. Heart rate variability did not significantly differ among treatment groups.

Table 1. Novel tank diving and blood glucose test results at 8 weeks during the pilot study.

Diet	Time Spent in Top Half (s)	Entries Into Top Half	Blood Glucose Level (mg/dL)
Balanced	33.136	1	39
Balanced	44.519	5	47
High Carb	0.985	0	42
High Carb	129.772	6	47
High Carb	119.121	12	67
High Carb	249.932	10	118

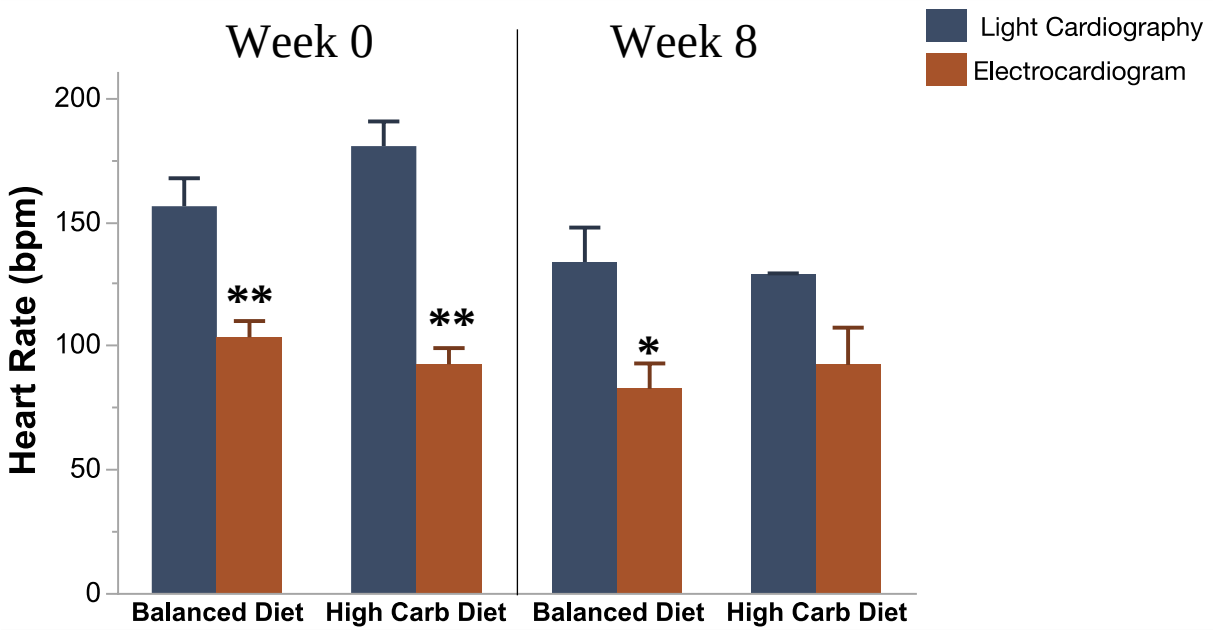

Figure 4. Heart rate measurements using light cardiography and electrocardiography at 0 weeks (Balanced diet = 12, High carbohydrate diet = 12) and at 8 weeks (Balanced diet = 5, High carbohydrate diet = 3) during the pilot study * and ** represents $p=0.018$ and $p<0.001$ respectively. Error bars represent ± 1 standard error.

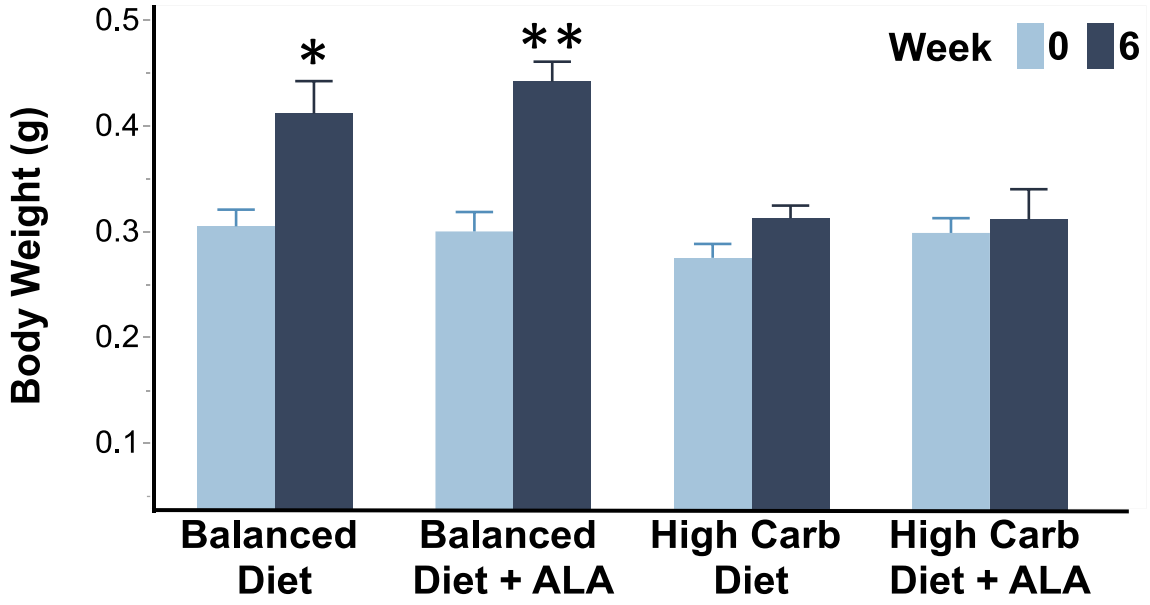

Figure 5. Body weight change over six weeks in control diet (No ALA = 15, ALA = 17) and high carbohydrate diet (No ALA= 15, ALA=13) zebrafish. * and ** represents p=0.0042 and p<0.001 respectively. Error bars represent ± 1 standard error.

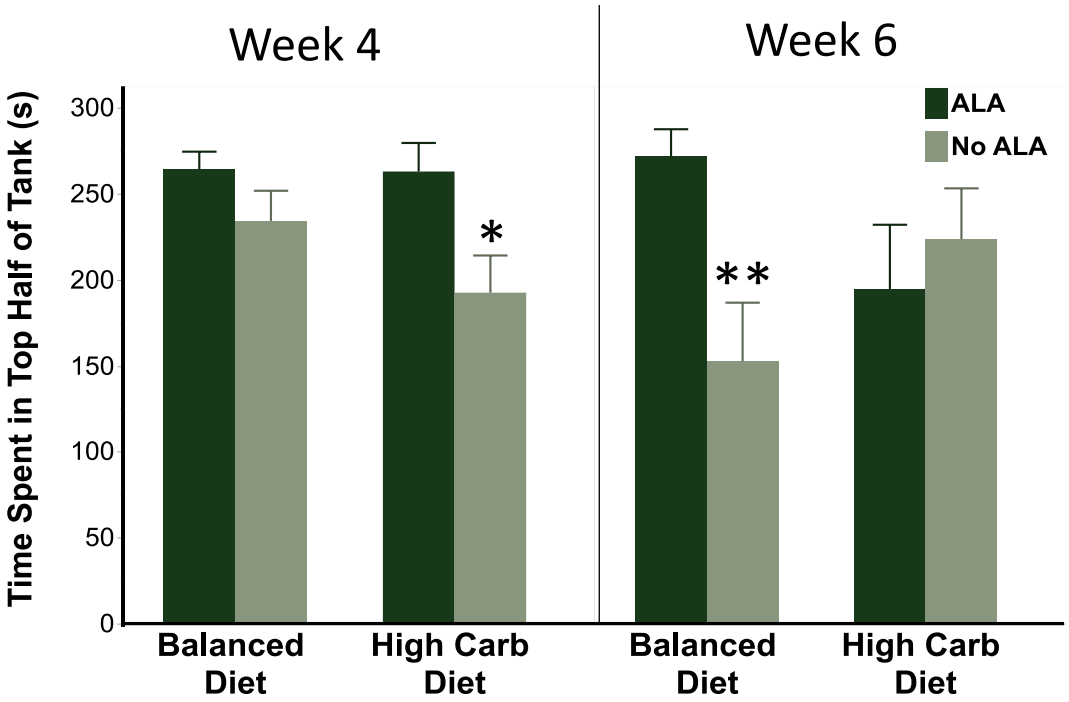

Figure 6. Novel tank diving results at 4 and 6 weeks in control diet (No ALA = 15, ALA = 17) and high carbohydrate diet (No ALA= 15, ALA=13) zebrafish. * and ** represents $p=0.0339$ and $p<0.0137$ respectively. Error bars represent ± 1 standard error.

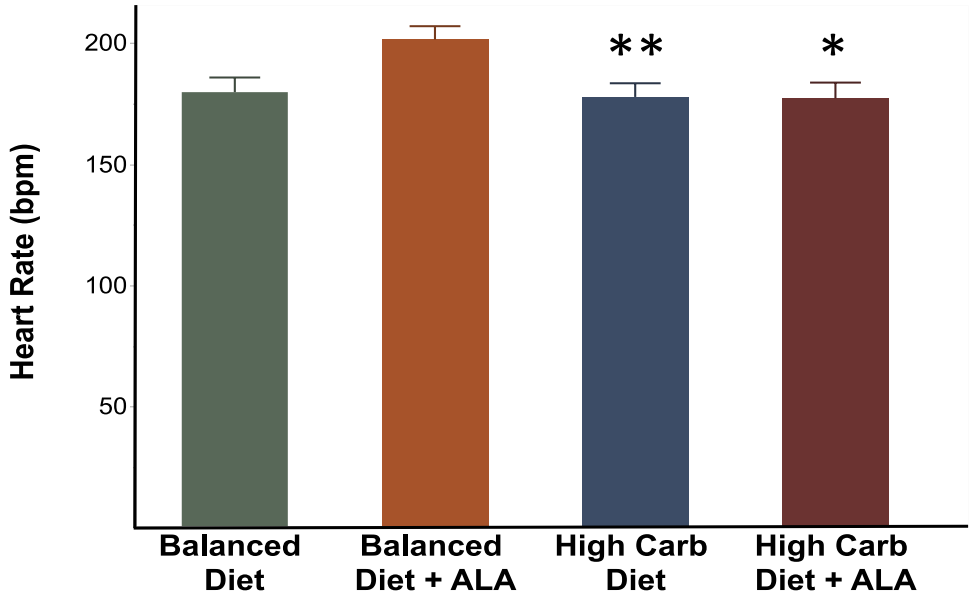

Figure 7. Heart rate from light cardiography at 6 weeks in control diet (No ALA = 15, ALA = 17) and high carbohydrate diet (No ALA= 15, ALA=13) zebrafish. * and ** represents $p=0.0202$ and $p<0.0114$ respectively. Error bars represent ± 1 standard error.

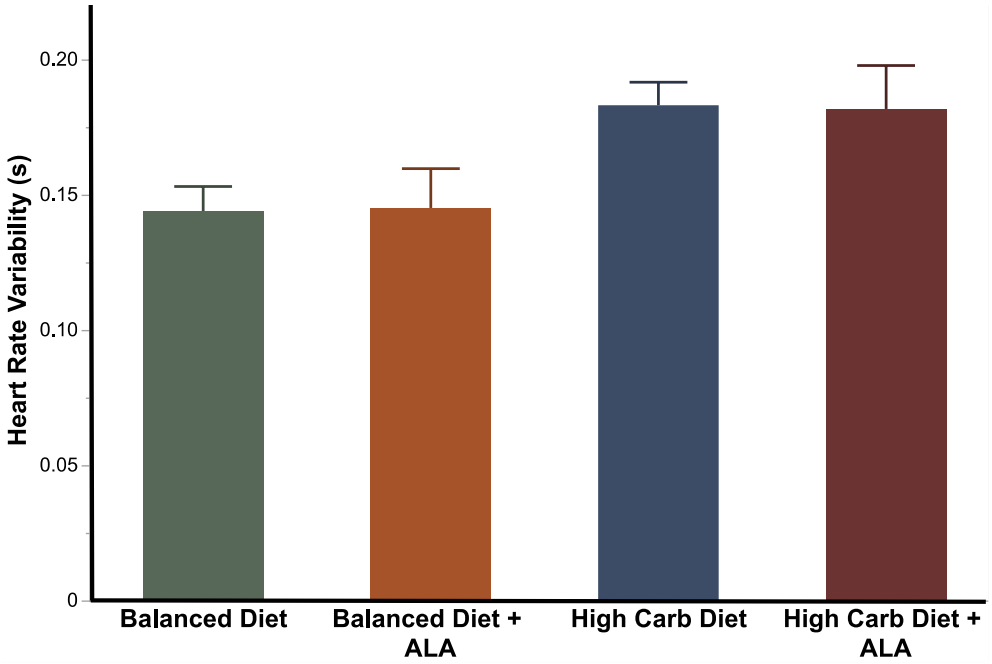

Figure 8. Heart rate variability from light cardiography at 6 weeks in control diet (No ALA = 15, ALA = 17) and high carbohydrate diet (No ALA= 15, ALA=13) zebrafish. Heart rate variability did not significantly differ between groups. Error bars represent ± 1 standard error.

V. Discussion

We used well established tests, ECG, and blood glucose test, to analyze the validity of less established tests (novel tank diving and light cardiography). The pilot established that electrocardiography (ECG), light cardiography and novel tank diving tests are all quantifiable, however the ECG technique is invasive and not ideal for repeated testing.

Previous studies have shown in an ECG recording after 3-5 minutes of anesthetization with 0.02% - 0.04% MS-222, 12–18-month-old zebrafish heart rate was around 116 ± 17 beats per minute (bpm) (Zhao et al., 2019). Before the diets start in this study the average heart rate from light cardiography recording was 169 bpm and 98 bpm from electrocardiography. After

eight weeks, a high carbohydrate did not affect heart rate measured by electrocardiography of light cardiography.

An electrocardiograph was done on zebrafish immediately after the first and last trial of light cardiography while the fish were still under anesthesia. Zebrafish were under anesthesia longer before electrocardiography was performed in comparison to time under anesthesia before light cardiography. In zebrafish anesthetized with MS-222, heart rate significantly decreases after five minutes of sedation (Lin et al., 2018). The prolonged time under anesthesia may explain the significantly lower heart rate during the electrocardiogram compared to light cardiography.

Blood glucose tests were attempted on eight total fish, but only six fish had successful tests done. Enough blood from the other two fish was not able to be extracted to get an accurate reading. The average adult zebrafish fasting blood glucose level is around 74 mg/dL and hyperglycemic zebrafish are above 200 mg/dL (Cao et al., 2023). The fasting blood glucose of the zebrafish in our pilot study ranged from 39-118 mg/dL The limited data points did not result in a significant difference in blood glucose level between high carbohydrate and balanced diet zebrafish after eight weeks. A larger sample size is needed to further evaluate the effects of this high carbohydrate diet on blood glucose concentration. Additionally using glucose immersion rather than incorporation into diet may have achieved higher blood glucose concentration as it has shown to induce chronic hyperglycemia in zebrafish within two weeks (Capiotti et al., 2014). Both time spent in the top half and entries made into the top half of the novel tank positively correlated with blood glucose level. This was unexpected as previous studies showed hyperglycemic zebrafish spent less time in the top half of the tank and less entries into the top half of the tank (Dos Santos et al., 2018; Wang et al., 2020).

Diet composition had a significant effect on zebrafish weight, but alpha lipoic acid exposure did not. Despite both groups being fed the same amount of food, only zebrafish fed a balanced diet had a significant weight gain. The lack of weight gain in high carbohydrate diet zebrafish may suggest type 2 diabetes mellitus developed in these fish. Inadequate cellular uptake glucose can cause unintentional weight loss in diabetics. This could explain the lack of weight gain in high carbohydrate diet zebrafish in contrast to the significant weight gain in balanced diet zebrafish.

Wang et al. (2020) found that hyperglycemic zebrafish exhibited anxiety like behaviors during a novel tank diving test. Hyperglycemic fish spent less time in the top half of the tank and made less entries to the top half of the tank. In our study, at 4 weeks of a high carbohydrate diet and at 6 weeks of a control diet, zebrafish that were concurrently treated with ALA spent significantly more time in the top half of a novel tank during testing. At 4 weeks, high carbohydrate diet fish not exposed to ALA made more entries into the top half of the novel tank compared to balanced diet fish exposed to ALA. This suggests ALA may have a protective effect against hyperglycemia induced anxiety-like behaviors in zebrafish.

At 6 weeks zebrafish fed a high carbohydrate diet with and without alpha lipoic acid exposure had a significantly lower heart rate then fish fed a balanced diet with alpha lipoic acid treatment. Since tachycardia is one of the symptoms of cardiac autonomic neuropathy, we were expecting high carbohydrate diet zebrafish to have an increased heart rate. The decreased heart rate may be a result of autonomic imbalance while under anesthesia. Cardiac autonomic neuropathy patients often become bradycardic under anesthesia (Lankhorst et al., 2015; Lee et al., 2023; Lourdes et al., 1989). Since zebrafish were anesthetized during light cardiography, the decreased heart in high carbohydrate groups compared to balanced diet groups may have been a

result of diabetic CAN induced autonomic imbalance. Performing light cardiography under anesthesia may also explain why heart rate variability did not significantly differ between groups. General anesthesia reduces HRV, so testing in awake zebrafish may be a more accurate reflection of heart rate variability (Matchett et al., 2014).

The results of this study may have been improved by housing the zebrafish in a glucose solution rather than feeding them a high carbohydrate diet. Previous studies have shown successful development of chronic hyperglycemia when fish were emersed in 111 mM glucose solutions (Capiotti et al., 2014). Additionally, only one round of novel tank diving and light cardiography done at the end of the study may improve the experiment. Since novel tank diving is a test of anxiety, repeated testing may hinder the results. Light cardiography requires anesthesia, and repeated sedation and recovery may affect heart rate.

In conclusion, six weeks of a high carbohydrate diet in adult zebrafish increases body weight and reduces heart rate despite ALA treatment. At four- and six-weeks ALA treatment reduces anxiety like behavior during novel tank diving in high carbohydrate diet zebrafish and balanced diet zebrafish respectively. To expand upon this study, additional research is needed to determine if a higher dose of ALA or longer treatment duration is needed to elicit an effect on diabetic cardiac autonomic neuropathy development.

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