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The Effects of Creatine Supplementation on Muscle Synthesis and Fitness Levels in Drosophila melanogaster Using a Model of Muscle Atrophy

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The Effects of Creatine Supplementation on Muscle Synthesis and Fitness Levels in *Drosophila melanogaster* Using a Model of Muscle Atrophy

By

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Abstract

This study examines whether an inhibited electron transport chain can be aided with supplemented creatine to make up for the challenged adenosine triphosphate (ATP) production mechanism. The electron transport chain is a series of protein complexes in the mitochondrial membrane that transfer electrons and couples this reaction with the transfer of protons across the membrane to produce ATP. The differences between male and female flies and the supplementation of creatine at a 0.15% concentration was studied. The relationship between these components was studied over the course of two 3-week trials using a fly treadmill and *Drosophila*. The effects of creatine on a mitochondrial disease modeled *Drosophila melanogaster* were analyzed by tracking climbing speed, leg width, and body width. The research yielded largely minimal differences between supplemented and non-supplemented, mutated, and non-mutated mitochondria, and before and after the trial. Combined gender trials showed qualitative decreases in leg width for mutated flies and a slight increase for wild type flies. Combining trials and genders yielded results that were largely inconclusive.

Keywords: *Drosophila melanogaster*, Creatine, Adenosine Triphosphate, ATP, Cytochrome Oxidase

Introduction

The supplement industry is a multi-billion-dollar industry relying on the goal of improving lives with manipulation of diet. The goal is to increase health and strength with the addition of a supplement. This is especially popular in the sports industry. Many athletes want to optimize their performance, and one of the most popular athletic supplements is creatine. Creatine is naturally synthesized by the body in the kidneys, pancreas, and liver, and is consumed in the diet predominantly by eating meats[1]. Creatine was discovered in 1832 but did not become commonly

used until 1992 when an athlete at the Barcelona Olympics brought the supplement to popular attention by telling the press he supplemented with creatine after he won his race[2]. This compound is involved in twenty metabolic pathways[3] including the electron transport chain.

Creatine has been widely recommended for natural performance enhancement because of how it helps make ATP regeneration more efficient[4] and increases lean body mass[5]. The positive effects on health can be expressed in[6] the way the body utilizes this to make a high energy compound to replenish its ATP stores outside of the mitochondria[7,8]. Creatine ingested or naturally synthesized travels to the mitochondria and is phosphorylated to become phosphocreatine (PCr). This phosphocreatine provides the phosphate group to the adenosine diphosphate (ADP) to become adenosine triphosphate (ATP). Cells use this high energy molecule in the body's energy consuming processes[7,8].

This process of metabolism involves oxidative phosphorylation. This occurs in eukaryotes in the inner mitochondrial membrane and relies on four complexes to create a gradient that ultimately powers complex V (ATP synthase) to create ATP. The purpose of this enzyme, which involves a head, base, and neck, is to provide ATP by catalyzing the last step in oxidative phosphorylation. The ATP synthesis process is coupled with ADP and creatine kinase to link to the high energy phosphate reservoir of phosphocreatine. Creatine kinase and ATPase kinetics are related and constant among different subjects, and it has been shown through enzyme kinetics that the four molecules, PCr, ATP, and P_i are closely associated in the exchange network to providing energy for the body[9]. This phosphocreatine also acts as a carrier for transferring energy from the mitochondria to the cytosol. Phosphocreatine can be used in the cytosol to prolong energy expenditure by providing the needed phosphate group to the produced ADP. The body has a limited storage of ATP. When the oxidative phosphorylation cannot keep

up during peak energy demands, the added supply of high energy phosphocreatine can supplement the production of ATP. Besides this benefit, creatine has also been shown to have antioxidant capabilities to reduce reactive oxygen species and a decrease in apoptotic enzyme activities in oxidative skeletal muscle[6].

A cytochrome C oxidase is the fourth complex in the electron transport chain, and it functions to oxidize cytochrome C by transferring electrons from the cytochrome C to oxygen[10]. It is thought that a mutation to this protein likely decreases the function of the electron transport chain and ultimately decrease cellular energy levels. Indeed, the symptoms from this complication in humans, like lethargy or lack of muscle tone, are known as Leigh Syndrome[11]. This mitochondrial disease of cytochrome C oxidase functions similarly in humans and *Drosophila*. *Drosophila melanogaster*, more commonly known as fruit flies, are a common model organism. Previous research has shown that the pathways involved with creatine in humans are similar to those in *Drosophila melanogaster*[8]. Genes control muscle size and survival, and the expression or suppression of certain genes can play integral roles in muscle wasting diseases[12]. Fruit flies as a model organism for human applications are well established[8,12]. The ability to study the effects of this substance over generations is irreplicable in human studies. Creatine's possible ability to decrease muscle atrophy has the potential for numerous clinical applications[7,13]. Phenotypic expressions of the mutation in *D. melanogaster* are a severely decreased lifespan, leg width, body width, and activity[12]. However, the effects of creatine on these phenomena have not been extensively studied. I hypothesize that creatine supplementation can ameliorate this phenomenon in *D. Melanogaster*. Supplementing creatine enhances a reservoir of phosphocreatine that could then be utilized in the associated exchange networks, especially when the diseased model has a mitochondrial mutation that puts stress on

the system. If an adequate amount of creatine is supplemented in a complex IV mutated *Drosophila melanogaster*, we predict that the lifespan, body width, leg width, and activity will increase as compared to non-supplemented.

There are currently phase three trials for creatine supplementation in patients with Parkinson's and Huntington's disease[14]. There is a lack of experiments involving the topic of mitochondrial impairment. Therefore, the purpose of this research is to find medical applications for the supplementation of creatine, especially concerning mitochondrial disease and other diseases with similar pathways and/or symptoms.

Materials and Methods

Flies

Flies are from the Bloomington Drosophila Stock Center of Indiana University. Wild type (6326) and a human disease model for mitochondrial complex deficiency disease, cytochrome oxidase mutation (81006), were used. The mutation is inherited by the maternal offspring,[15] therefore the cross in figure 1 is necessary to ensure that only the mitochondrial DNA is different between flies with functional cytochrome C oxidase (Wt.) and those with the mutation (Mt).

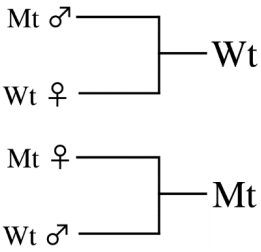

Fig. 1 Mitochondrial mutant male from stock crossed with wild type female from stock to create the experimental wild type fly with 50/50 nuclear DNA and 100% wild type mitochondrial DNA (Wt). Mutant female from stock crossed with wild type male from stock to create the experimental mutant fly with 50/50 nuclear DNA and 100% mutant mitochondrial DNA (Mt).

Housing

Humidity was kept between 60-70% via a water pan in an incubator set at 25˚C[16–20]. Lights on a 12-hour cycle kept the flies on a regular circadian rhythm. Flies were stored in glass *Drosophila* tubes with 2.3 grams of media per tube. Creatine (2-[Carbamimidoyl(methyl)amino]acetic acid)

produced by Iovate Health Sciences Inc. was mixed into the nutrition media of the flies. The medium contained creatine at concentrations of 0%, 0.15%, and 0.30% by weight. FlyNap was used to tranquilize the flies for sorting and measuring.

Exercise

Flies were exercised with an apparatus known as a "fly treadmill",[21] which changes which direction is "anti-gravity" in a non-invasive manner at one revolution per minute. The fly treadmill was made by the engineering department of Olivet Nazarene University and has the capacity for 24 *Drosophila* tubes with the capabilities to remotely view and control the exercising of the flies. Exercise consisted of rotation for ten minutes then rest for five minutes repeated three times in a row. This occurred five days a week for three weeks (Sunday and Wednesday were rest days).

The incubator housed the exercise apparatus ("fly treadmill") as well as the flies not exercising. The flies were separated by gender to avoid distraction[14]. Flies have a natural anti-gravity response (climbing to the top of a tube against gravity)[13]. This intrinsic motivation was utilized to exercise the flies by rotating the flies in tubes to induce climbing behavior. Flies from the same generation to ensure same age were exercised in *Drosophila* tubes (ten flies per tube for each trial) without food to avoid distraction.

Data Collection

To measure the fitness of the flies, a line was drawn on the tubes to mark two-thirds of the length of the tube and the amount of time it took half of the flies to cross it. Using a 12 megapixel, $f/1.6$ aperture camera and/or in-person visual monitoring, the amount of time until 50% of the flies climbed two-thirds (past the line) of the tube after being tapped to the bottom was recorded as a measure of tracking activity. To measure muscle deterioration, physical size of the thickest part

of the flies' body and one leg (femur width) was measured using a 12 megapixel, $f/1.6$ aperture camera and image J software, before and after the experiment. The resolution varied between 37 and 57 pixels per millimeter depending on how close the camera was to the flies. GraphPad Prism version 9.1 was used to create graphs and to perform statistical analyses.

Results

Leg Width

To evaluate the impact of creatine on muscle size, we measured the flies before and after the duration of the experiment for leg and body width. The flies use their legs to climb up the tubes, so leg width muscle change was observed. Using a one-way paired t-test, Male: Mt vs. Wt legs have p-values all above 0.05, which implies that there is not strong enough evidence to suggest an effect. The female legs show a decrease from before to after, with p-values over 0.05. Combining data from both trials and genders for legs resulted in statistically insignificant p-values, illustrated in figure 2. There is insufficient evidence to conclude that supplementation of creatine influences fly width or that a mitochondrial mutation of the cytochrome oxidase complex will yield different fly leg widths.

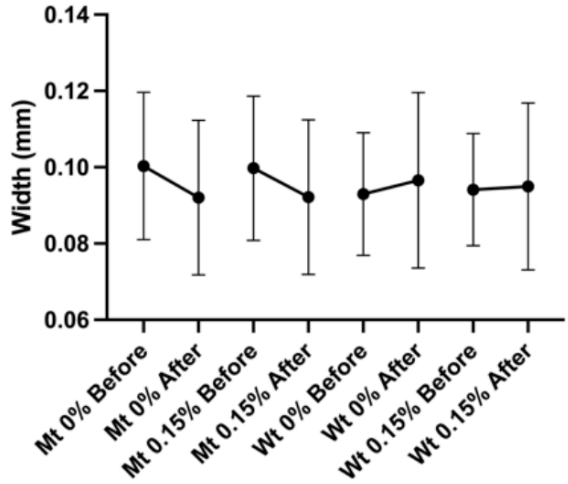

Fig. 2 Leg width is unchanged in exercise wild-type and cytochrome C oxidase mutant flies either with or without creatine. "Before" indicates the width measured at the start of the experiment, while "after" indicates the width measured after the experiment. Mean widths (average of all points in the study) are plotted with standard deviation error bars. Mutated fly leg width decreased regardless of concentration. Genders were separated for the trials, but the data from these trials was combined for this chart. Data are from two trials (Mt 0% n=70; Mt 15%, n=60; Wt 0%, n=46; Wt 0.15%, n=52)

Body Width

General size for flies was tracked measuring body width. Using paired t-tests, the values of combined genders and trials for body widths resulted in statistically insignificant values (p-value > 0.05). There is insufficient evidence to conclude that there is an increase or decrease in width for fly bodies with or without the supplementation of creatine or addition of a mutation to the mitochondrial cytochrome oxidase.

Figure 3 illustrates the different body measurements. This breakdown gives more insight into the results of the body study. Male and female flies were examined separately, but no relationship was observed. The first trial of female body widths did not yield relevant results when the measurements for all the flies were averaged. The second trial for female body width resulted in a difference in wild type flies before and after three weeks with no creatine in their nutrition. The genders were studied separately to obtain accurate data, then the data was combined for presentation and evaluation together.

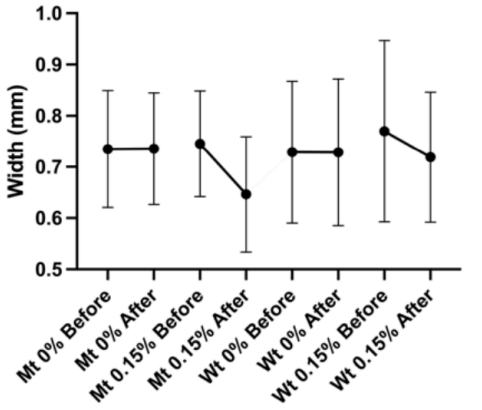

Fig. 3 This graph shows the first and second trials of body measurements for male and female flies, all combined. Data are from two trials (Mt 0% n=35; Mt 15%, n=30; Wt 0%, n=23; Wt 0.15%, n=26)

Climbing Time

Climbing time is used to quantitatively measure and represent the energetic and metabolic states of the flies. Qualitatively, WT in 0% creatine have on average quicker climb times compared to MT flies in 0% creatine, with overall average climb times of 3.55 seconds and 16.24 seconds, respectively. The graphs below exhibit these data points.

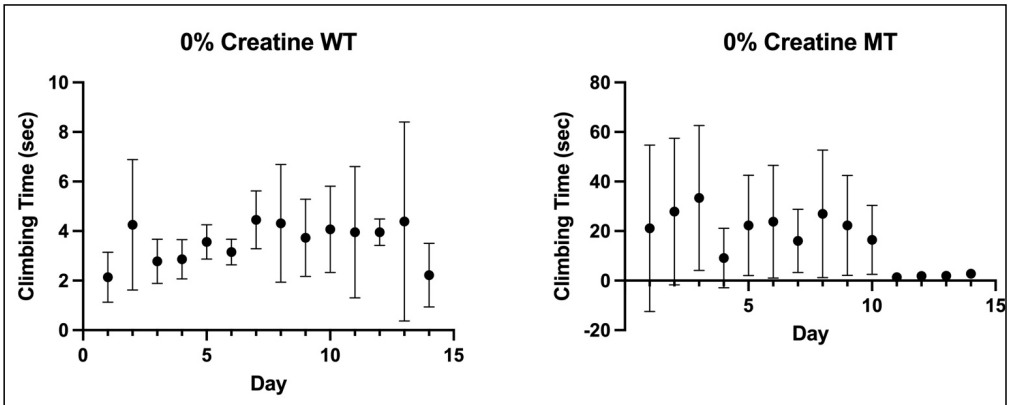

Figure 4a-b Gives the combined graphs of climbing times for both WT and MT flies in media with 0% creatine concentrations. Each point on the graph represents an average of trial 1 and trial 2 climbing times. This implies that each point contains eight separate climbing times averaged, as each gender was in separate tubes in duplicates for two trials. Each trial was averaged separately and those averages were then averaged to create the data points in **Figure 4a-b**. The x-axis indicates the day the flies were exercised during the three-week period. Note that in **Figure 4b** (right) days 11-14 reflect the data average of only tubes from trial 2 due to the death of flies from trial 1. Climb times were obtained once half of the flies in the tubes went past the marked 2/3 position.

Qualitative analysis of the graphs reveals a relatively similar relationship between 0.15% creatine WT climb times (Figure 5a, left) and 0.15% creatine MT climb times (Figure 5b, right), and overall average climb times per trial and designation indicate that WT was slightly slower at 8.34 seconds compared to 8.08 seconds for MT flies. Also note that it appears that days 1-2 for 0.15% creatine WT (Figure 5a) are outliers, bringing up the overall climbing time.

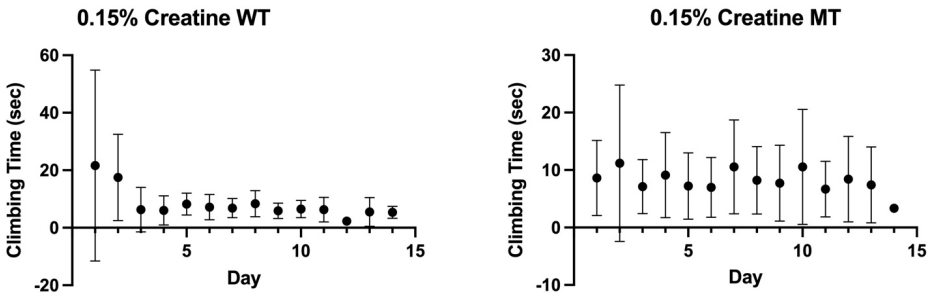

Figure 5a-b Gives the combined graphs of climbing times for both WT and MT flies in media with 0.15% creatine concentrations. Each point on the graph represents an average of trial 1 and trial 2 climbing times. This implies that each point contains eight separate climbing times averaged, as each gender was in separate tubes in duplicates for two trials. Each trial was averaged separately and those averages were then averaged to indicate the data points in **Figure 5a-b**. The x-axis indicates the day the flies were exercised during the three-week period during a trial. Climb times were obtained once half of the flies in the tubes went past the marked 2/3 position.

Discussion

We hypothesized that if creatine was supplemented in a mitochondrial disease modeled *D. melanogaster*, then the muscle would hypertrophy or show less atrophy and that the flies would have faster climbing rates when compared to non-supplemented. The results would show whether supplementing creatine is effective in offering a high-energy molecule reservoir. It is statistically concluded that the hypothesis can neither be accepted or rejected. The results from this research are less definitive than what was found in other research in terms of significance.

Leg Width

The findings from this section did not align with our hypothesis but did not oppose it either. Kreipke and Smith's articles described how muscle would hypertrophy with the presence of

creatine[7,13], yet the results found in this study did not seem to align with this. By looking at the graphs qualitatively, the female widths tend to all decrease through the duration of the trial regardless of genotype in the first trial, whereas male leg widths are all sporadic. The trials intended to show if the supplementation of creatine was able to increase the muscle mass in the presence of a disease model and provide more support for the ability to increase muscle size in a wild type individual. Though the concentrations used were determined by studying the literature (0%, 0.15%. 0.3%), it may have been beneficial to extend the number of concentrations studied to have smaller incremental changes from 0-0.30%.

Body Width

Examination of the data suggests that for both mutated and wild typed male body widths there is a decline in size over the course of a three-week timeline; however, this is not statistically proven. For female flies this is reflected for creatine concentrations of 0.15%; for male and female, but not for both genders, with creatine concentrations of 0%. When male and female body widths are combined, there appears to be no change with 0% creatine regardless of genotype, but there is a decrease for both genotypes with 0.15% supplemented creatine. When looking at the differences between mutants and wild types, there was no significant difference in males even qualitatively. Mutant flies have previously been known to have muscle atrophy[7]. This does not align with Garcia's[22] results, as there was no evidence that the phosphocreatine was helpful in establishing an energy reserve. The findings from the body width portion of this research neither supported nor opposed the hypothesis. The decision to measure the femur comes from past research. Other protocols measure wing muscles; however, flies use their legs to climb the treadmill, so it makes more sense to measure the muscles being activated with our research model.

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Climbing Time

The effects of cytochrome oxidase manipulation were inconclusive in the climbing time trials as well. Wild type flies had generally faster times than mutated flies, which was expected due to the hypoactivity effects of the mutation. The average time for 0.15% supplemented flies was lower than 0%; however, the difference is not statistically significant, which could indicate the compensating effect of creatine. The longitudinal portion of this study was affected by the lifespan of the flies; the un-supplemented mutant-flies from the first trial were all dead by day 11. A decreased lifespan was expected from the inhibiting mutation. Flies of this same type survived until the end for the second trial; the cause of this is unknown. Although inconclusive, information was obtained that can contribute to better results if the project was to be replicated. These things, like appropriate creatine concentrations, can be used in future experiments.

Conclusion

The purpose of this project was to test whether creatine could help negate the negative effects of a cytochrome C oxidase mutation. The results showed that this specific research does not provide conclusive results on the effects of this supplement. Other research has shown a more statistical difference between mutated cytochrome C oxidase and wild type flies[15]. There were many factors that contributed to the reliability and validity of this experiment. Time was a large factor, as the flies took longer than expected to be delivered and there was thus only enough time for two trials. Having only one stock tube of the wild type and one of the mutant necessitated us to collect flies as they hatched. This slowed the process and might have contributed to size variability, as the flies were then at different stages of development and could have been different sizes yet were all considered to be equal at the first day of the trial. The flies were all within thirty-six hours of age at the start, which was intended to synchronize fly age to the best

of our ability. The rotation speed of the treadmill needed to be lowered, as the pre-trial set of exercises left many flies dead and the rest were flying spastically at the bottom of their tubes. We do not know the implications, if any, that this trauma may have imposed, so the rotation speed was decreased and the flies reacted much better to this.

Metabolic similarities between humans and fruit flies indicate the fruit fly should have been a good model for studying creatine in this experiment. It is unknown why the mutant flies did not atrophy in every experiment. The Bloomington fly stock website indicated there was temperature sensitivity to the phenotype of the mitochondrial mutant flies, so the flies were stored at the appropriate temperature in a temperature-controlled incubator to ensure that the temperature was always suitable. Male and female flies were examined separately to ensure the flies did not reproduce. This separation also enables separate examinations. The photo quality for J image could have been higher. The resolution at points could have been better to get more accurate measurements. Overall, the project has potential to still produce relevant data. Furthermore, if these changes were made for future trials, the chances of significant results would be much higher.

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