Vol 1, Issue 3, pages 1-14 ***High School Research Journal***

USING ANTIOXIDANTS TO COUNTERACT MITOCHONDRIAL DYSFUNCTION AND ALPHA-SYNUCLEIN AGGREGATION IN PARKINSON'S DISEASE

Elin Hu and Vanessa Zhang*
Manhasset High School, New York

ABSTRACT

This study aimed to use antioxidants as potential therapeutics for mitochondrial dysfunction and α-synuclein aggregation. It was hypothesized that mitochondria-targeted antioxidants, MitoQ (1 and 5 μM) and MitoTEMPO (10 and 50 μM) will decrease mitochondrial dysfunction in *C. elegans* and Vitamin C (80 uM) and Curcumin (80 uM) will decrease effects of a-synuclein aggregation in *Drosophila melanogaster*. In aspect 1, mitochondrial pathways of Parkinson's disease, the overproduction of ROS and complex malfunction, were assessed. Lead nitrate exposed *C. elegans* expressing GFP in the nuclei and mitochondria of body wall muscle cells were assessed in locomotion and chemosensation. It was found that exposure to MitoQ and MitoTEMPO greatly increased locomotion and chemosensation. Fluorescence microscopy was used to study mitochondria in *C. elegans* and results were quantified using Image J software. MitoQ and MitoTEMPO greatly increased the fluorescence of Parkinson's disease affected *C. elegans*. In aspect 2 Drosophila expressing GFP fluorescence in dopaminergic neurons, mutant α-synuclein in dopaminergic neurons, and lead nitrate exposed Drosophila expressing GFP fluorescence in dopaminergic neurons were tested in their sleep behavior and climbing ability. When exposed to Vitamin C and Curcumin, the climbing ability of mutant α-synuclein and lead nitrate exposed Drosophila was significantly increased and their sleep behavior was returned to normal ($p<0.05$). Fluorescence microscopy was used to study dopaminergic neurons in Drosophila and results were quantified using Image J software. It was found that Vitamin C and Curcumin significantly increased the fluorescence of dopaminergic neurons in Parkinson's disease model Drosophila.

Keywords: Parkinson's disease; Mitochondria; α-synuclein; MitoQ; MitoTEMPO; Vitamin C; Curcumin

INTRODUCTION

Parkinson's disease is the second most common degenerative of the central nervous system [1]. Up to 1 million people in the U.S. alone are affected with Parkinson's disease and

*Corresponding Author address
Email: nile820@gmail.com

as many as 60,000 cases are diagnosed by doctors each year [2]. The number of deaths associated with Parkinson's disease is projected to continue increasing in the future.

Parkinson's disease is characterized by the neurodegeneration of dopaminergic neurons in the substantia nigra of the brain. The symptoms that arise with Parkinson's disease are most commonly motor deficits such as bradykinesia and tremor [3]. Other symptoms include loss of memory and decline of speaking ability and cognitive function. Another prevalent non-motor symptom that can occur is abnormal sleep activity cycles [4]. Currently there is no cure for PD as the definite cause is not yet certain, but there are treatments that delay neurodegeneration and the development of symptoms.

Parkinson's disease models show death of nerve cells that produce dopamine, which in turn causes dopamine deficiency [5,6]. Though the actual cause of Parkinson's disease is unknown, studies suggest that the dopaminergic neuronal death that causes the disease could result from a misfolded α-synuclein protein, mitochondrial dysfunction, or inflammation in the brain [5,7,8,9]

These factors detrimentally affect dopaminergic neurons. In normal dopaminergic neurons, action potentials are passed from the dendrites to the axon terminals. At the resting potential, sodium ions are concentrated outside the cell and potassium ions are concentrated inside the cell. As an action potential is passed down the axon, sodium ion channel gates open and allow sodium ions to enter the cell, which causes depolarization. Potassium ions are then released from the cell, which causes repolarization. This repolarization then prompts voltage gated calcium ion channels to open and allow an influx of calcium ions. When calcium ions enter the cell, vesicles containing dopamine are stimulated to release dopamine into the synapse, which bind to the post-synaptic receptors and transmit the message to the next neuron [10]. However, mitochondrial dysfunction and α-synuclein aggregation can disrupt the transmission of messages by causing the degeneration of dopaminergic neurons. Mitochondrial dysfunction and α-synuclein aggregates cause microglial cells, hypersensitive immune cells, to overproduce reactive oxygen species, which leads to oxidative stress in the dopaminergic neurons and subsequent neurodegeneration [11].

Another factor that can cause parkinsonism is the misfolding and aggregation of α-synuclein. α-synuclein is usually bound to the membrane of the presynaptic termini of neurons, but sometimes α-synuclein will detach from the membrane and misfold [12]. α-synuclein will misfold into oligomers that form layered fibrils, which can accumulate to form Lewy bodies. Lewy bodies are detrimental to dopaminergic neurons because they can block the transport of vesicles containing dopamine, which prevents the release of dopamine into the synapse [13]. The α-synuclein aggregates that form Lewy bodies can also cause microglial cells to overproduce toxic reactive oxygen species, leading to oxidative stress and the death of dopaminergic neurons [11].

Mitochondria are an important source of ROS, the production of which results in mitochondrial damage in a range of pathologies [14]. The electron transport chain is a system made of electron carrier complexes found in the mitochondrial inner membrane, between the intermembrane space and the matrix. Three complexes are involved in this system. Complex 1, or NADH dehydrogenase, reduces NADH into NAD+ and two electrons are transported from complex 1 to ubiquinone. Electrons flow from complex 1, to ubiquinone, an organic molecule that can move freely within the hydrophobic region of the membrane, and then to complex two, or cytochrome reductase. As NADH is reduced, protons (H+) are pumped across complex 1, creating a proton gradient. Cytochrome C, a small protein, accepts the electrons one by one and transports them to complex 3, or cytochrome oxidase. Using 4 electrons, 8 protons, and molecular oxygen, Complex 3 releases two water molecules into the matrix and releases the excess protons, further establishing the gradient. Along the chain, especially with complex deficiencies, electrons can leak and reduce oxygen (O2) to the free-radical species, superoxide. Superoxide is extremely reactive and form potent oxidants or radicals, overall increasing ROS levels and leading to oxidative stress [15].

Oxidative stress is a major implicated factor in the development of neurodegeneration in PD pathogenesis. Oxidative stress, which can result from overproduction of POS or deficient ROS removal, can ultimately damage cellular lipids,

proteins, and DNA. Mitochondrial dysfunction is not only the main factor that increases oxidative stress in PD, but can also be exacerbated by oxidative stress [16].

A non-motor symptom that has been found to accompany other Parkinsonian symptoms is an abnormal sleep cycle [4]. Mutant A53T α-synuclein Drosophila exhibit pre-fibrillar α-synuclein oligomers, which will cause a dysfunctional neuronal system and disrupt the circadian rhythm of the Drosophila. These mutant Drosophila have been shown to exhibit more excessive sleep during the daytime than wild type Drosophila and a shift in their sleep cycle.

Environmental factors also can contribute to the rise of Parkinson's disease. Lead nitrate has been shown to cause deficits in neurotransmission. Lead exposure impairs excitatory postsynaptic currents (EPSC's) and inhibitory postsynaptic currents (IPSC's). These currents depend on neurotransmitter release from the presynaptic neuron, so decreases in EPSC's and IPSC's show a deficit in neurotransmission across the synapse. This could be because lead inhibits the presynaptic neuronal voltage-gated calcium channels and this can prevent the necessary rise in internal Ca^{2+} needed for the vesicular release of neurotransmitters [17]. In addition to causing deficits in neurotransmission, lead nitrate has been shown to induce oxidative stress by increasing ROS generation and by altering antioxidant activities that regulate ROS [18].

Ng et al. found, in a study performed in 2014, that the mitochondrial targeted antioxidant MitoQ protected *C. elegans* models of Alzheimer's disease against oxidative stress. *C. elegans* treated with MitoQ had significant mean lifespan extension of 14% compared to the untreated control animals while its parent antioxidant, dTPP, did not extend mean lifespan. Impact of MitoQ and dTPP on electron transport chain activity was also observed by measuring fluorecence of complex I NADH. A significantly higher complex I activity level was detected in MitoQ-treated *C. elegans* [19].

In 2012, Johnson et al. found that mito-TEMPO significantly reduced the redox environment in the matrices of *eat-3* mutant *C. elegans* mitochondria. *C. elegans* were exposed to both the antioxidant compound, TEMPOL, and its mitochondria targeted derivative, mito-TEMPO and mitochondrial redox status was examined. It was demonstrated that specifically relieving and targeting mitochondrial oxidative stress protects *C. elegans* from ROS overproduction and abnormal mitochondrial redox [20].

Phom et al., in 2014, found that curcumin successfully rescued Drosophila against mobility defects induced by paraquat, a pesticide that causes parkinsonian symptoms. A negative geotaxis assay was performed on Drosophila exposed to both paraquat and curcumin to test the motor functions of the Drosophila. This was compared with the locomotion of Drosophila only exposed to paraquat. It was found that the Drosophila fed with curcumin did not have apparent movement defects and the speed of the flies was significantly improved when compared to paraquat-treated flies. High performance liquid chromatography (HLPC) was used to quantify brain dopamine levels and it was found that deplenished brain dopamine levels from paraquat exposure were replenished from curcumin treatment [21].

Khan, in 2012, found that Vitamin C ameliorates the effects of alpha synuclein aggregates in *Drosophila melanogaster*. The exposure to Vitamin C showed protective effects and delayed the loss of climbing ability of the Drosophila model of Parkinson's disease that expressed mutant alpha synuclein, which shows that the antioxidant nature of Vitamin C can help protect against the symptoms of Parkinson's disease [22].

The purpose of this study was to ameliorate the parkinsonian effects from mitochondrial dysfunction and α-synuclein aggregation. It was hypothesized that curcumin (80 uM) and vitamin C (80 uM) will decrease neurodegeneration and allow for increased motor function and regular sleep activity in Drosophila melanogaster models of Parkinson's disease and a mitochondria targeted antioxidant, MitoQ in concentrations of 1 and 5 μM and MitoTEMPO in concentrations of 10 and 50 μM will decrease mitochondrial dysfunction due to ROS and act as a DA neuro-protective agent in *Caenorhabditis elegans*.

METHODS

Aspect 1 (Mitochondrial dysfunction)

C. elegans Care and Maintenance

Strain CB5600 was be obtained from the CGC and cultured on nematode growth medium plates with OP50 E. coli as a food source.

Preparation of NGM plates: Equipment and Reagents

Bottles of NGM were obtained from Carolina Biological. A bottle was placed in the microwave with the cap off and heated at 10 second intervals until completely melted. The NGM was then distributed into petri dishes (35x10mm) using a serological pipette to insure accuracy and half covered with a lid to harden and cool. The plates were then stored upside-down at room temperature to prevent condensation on the surface of the agar.

Preparation of OP50 bacteria

Luria Broth base was used to make the culturing broth for E. coli. The base was added to water at the appropriate concentration according to volume and mixed thoroughly over a hot plate to eliminate clumps. The broth was then autoclaved to sterilize and distributed into sterile culture tubes and lead nitrate was added to achieve a concentration of 2.5μM $PbNO_3$.

Preparation of E. Coli OP50

A starter culture of E. coli OP50 was obtained from the CGC. Culture tubes and caps were sterilized in the autoclave, along with the luria broth. To make the lead nitrate treated OP50, a concentration of 2.5 μM was obtained with the lead nitrate and luria broth. The mouths of the tubes were sterilized over a Bunsen burner flame along with an inoculating loop, which was used to obtain one well defined colony from the plate culture. The loop was inserted into the broth and stirred to dislodge the culture. The top and the loop were reflamed and the tube capped. The culture was placed in an incubator to promote bacterial growth.

Seeding NGM plates

To seed plates, a sterile cotton applicator was inserted into the OP50 culture and the tip swabbed back and forth across the surface without touching the sides. The plates were then incubated at room temperature overnight for the lawn to grow and stored upside down until used to prevent condensation.

Worm Picking

3 cm of wire was cut and placed into a pasteur pipette and sealed into the glass over a bunsen burner. To pick worms, the wire was sterilized on a flame and dragged across the bacterial lawn to coat the surface with bacterial. Under a microscope, the tip was lightly brushed against a C. elegan without damaging or poking the agar surface. Once the worm was on the tip, it was be transferred to the new plate by tapping it to the surface.

Chunking Worms

A sterilized scalpel was used to move a chunk of agar from an old plate to a fresh plate, but cutting a small square of agar with plenty of worms.

Preparation of worms on slides

C. elegans were washed three times using the M9 buffer and then transferred to glass slides with sodium azide to paralyze the c elegan and then enclosed with a coverslip. They were put under the fluorescent microscope.

Age Synchronization using Bleach:

C. elegans were chunked onto a seeded NGM plate and allowed to grow for 2 to three days, until there were plenty of eggs. Once there are plenty of eggs, 5mL of M9 buffer was poured onto the plate and gently spun to dislodge the C. elegans. Using a pipette, the C.

elegans were then transferred into a conical centrifuge tube and centrifuged for 1 minute on high to pellet them. Most of the M9 buffer were aspirated without disturbing the worm pellet. Then, 5 mL of 20% bleach solution was added to the tube. The tube was gently mixed for 5 minutes by gently inverting. Once most of the bodies dissolved, the C. elegans were centrifuged on high for one minute to pellet and most of the solution was aspirated, leaving the pellet. M9 was added and the tube mixed and centrifuged again on high for one minute. This process of washing the C. elegans with M9 buffer was repeated 2-3 times. Fresh M9 was then added to re-suspend the pellet and the eggs were allowed to hatch overnight with gentle rocking from an orbital shaker. The liquid was then spread onto a seeded NGM plate.

Body Bending Aptitude

Worms of varying stages were assessed for the number of sinusoidal motions completed in a minute beginning from day one L1 stage worms to day 3. A nematode was picked onto the surface of a 35mm agar plate. Before performing the assay, the nematode was allowed to crawl freely to remove any bacteria from the worm. The number of body bends (motion that swings the head of the worm to the opposite side) was quantified for 1 minute.

Chemotaxis

Preparing the Test Plates

The underside of a 5 cm NGM plate was divided into 4 equal quadrants and a circle of radius 0.5 cm was marked around the origin. Each quadrant was labeled with either a "T" for "Test" or a "C" for "Control," ensuring that the sites were equidistant from the origin and each other. The points were 2 cm away from the origin. The top left and bottom right quadrant were marked as test quadrants and the top right and bottom left quadrants as control.

Running the Assay

The test solution comprised of salt water that was used as an attractant or ethanol as a deterrent. 2 uL of age synchronized worms from the pellet were pipetted onto the origin and immediately after, 2 µL of the paralyzing agent (40% ethanol) was pipetted onto each of the four points, and 2 µL of the test solution (1 mM NaCl) was pipetted onto the points labelled “T.” Once the worm and odorant drops were absorbed by the agar, the lids were replaced, and the plates inverted. After 60 minutes, the number of worms in each quadrant that completely crossed the inner circle were counted and recorded. Chemotaxis index was calculated using the equation [Chemotaxis Index= (# Worms in Both Test Quadrants- # Worms in Both Control Quadrants)/ (Total # of Scored Worms)]. This value should be between -1.0 and +1.0. A +1.0 score indicated maximal attraction towards the target and represented 100% of the worms arriving in the quadrants containing the chemical target. An index of -1.0 was evidence of maximal repulsion.Chemotaxis Index (CI) was reported along with standard deviation. A Student’s T-test was performed to compare the data of test and control plates.

Fluorescent analysis of GFP in mitochondria of body wall muscle cells

Fluorescent pictures of adult CB5600 *C. elegans* were taken under a Zeiss Axiovert fluorescent microscope at 400x magnification and then analyzed using Image J. A 525-550 BP emission filter was used to view the fluorescence of the GFP in mitochondria.

Statistical Analysis

Data was analyzed using the SPSS software. Data for fluorescence and chemotaxis was analyzed using a One way ANOVA followed by a post hoc Scheffe test ($p<0.05$). Data for body bend frequency was analyzed using a five by three mixed ANOVA followed by Tukey and Games-Howell post hoc tests ($p<0.05$).

Aspect 2 (Alpha synuclein aggregation)

Drosophila Strains

All Drosophila strains were obtained from Bloomington Stock Center. Expression of green fluorescent protein (GFP) in Dopaminergic neurons was achieved by crossing flies

homozygous for both UAS:mCD8-GFP and TH-GAL4. UAS-HsapSNCA.F Drosophila that express wild type human alpha-synuclein under UAS control were also used. After crossing, the Drosophila expressing GFP in dopaminergic neurons were exposed to Lead Nitrate (0.5 mg/mL).

Drosophila maintenance

Drosophila stocks purchased from Bloomington stock center were anesthetized by placing for ~2 min in a freezer. New empty vials were prepared with 6 ml of drosophila 4-24 medium and 10 ml of bottled water. A pinch of yeast was added to the medium. The anesthetized drosophila were then be moved from the old stock to the newly made vial. This protocol serves to keep past flies alive and to synchronize the age of the flies (CSH protocols, 2011).

Sexing Drosophila to obtain Virgin Females

Flies were anesthetized by placing them in the freezer for ~2 minutes. Anesthetized Drosophila were then transferred onto an ice pack to maintain their anesthetized state. Using a soft brushed bristle, flies were separated through examination of biological differences under a microscope. Male flies and female flies are differentiated since male torsos are surrounded by heavy dark bristles which do not occur on females. Female flies also exhibit a darker abdomen with a striped posterior. Male flies are also considerably smaller than the female flies and exhibit a darker posterior part with no stripes. Virgin drosophila were collected within 8-12 hours after the culture has been cleared of adults, since Drosophila do not mate for about 12 hours.

Crossing Drosophila Strains [23]

The strain expressing GFP that were purchased from the Bloomington stock center is under UAS (upstream activating sequence) control, which means that the gene for GFP were not expressed yet. To express the gene, the drosophila were crossed with the TH-GAL4 strain, which allowed GFP to only be expressed in the dopaminergic neurons. The alpha synuclein Drosophila expressed under UAS control were also crossed with the TH-GAL4 strain. The Drosophila expressing GFP in dopaminergic neurons were then crossed with the crossed alpha synuclein Drosophila, so that GFP was expressed in the dopaminergic neurons of the alpha-synuclein Drosophila.

Exposing Drosophila to Lead Nitrate, Curcumin, and Vitamin C

Lead nitrate was added to Drosophila medium of the group of Drosophila that express GFP in dopaminergic neurons at 0.5 mg/mL. Curcumin and Vitamin C were added to Drosophila medium at 80 uM.

Dissection of Adult Drosophila Brains [24]

Under the microscope, the thorax of the Drosophila was grabbed below the head so that the legs and proboscis extended. The proboscis was grabbed with the forceps to remove the head from the body. The head was then submerged in the PBS buffer solution in the depression slide and focused on under the microscope. The connective tissue between the proboscis and the eye was then torn and then the retina of the eye was torn away while working towards the back of the head. Continuing to the back of the head, the cuticle was torn away and the other eye was torn through. Once the cuticle was pulled away and the brain was visible, the trachea that is attached to the brain was removed. Finally, any more cuticle and trachea was removed to fully isolate the brain.

Fluorescent analysis of GFP in Dopaminergic neurons

Immediately following brain dissection, pictures of adult Drosophila brains were taken under a Zeiss Axiovert fluorescent microscope at 400x magnification and then analyzed using Image J. A 530-565 BP emission filter was used to view the fluorescence of the GFP in dopaminergic neurons.

Negative Geotaxis Assay

This assay was prepared by joining two vials together vertically and measuring 8 cm on the lower vial and drawing a circle around the circumference. The flies were then transferred into the lower vial and allowed to acclimate for 1 minute in the vial. Then they were gently tapped to the bottom of the vial and the number of flies that pass 8 cm line within 10 seconds was measured.

Sleep Assay

Drosophila from each group were placed into separate Drosophila Activity Monitor (DAM2) vials. The vials were prepared by putting drosophila medium into one end of the vial and then placing one anesthetized drosophila into the vial and putting more drosophila medium in at the opposite end. Then the vials were put into the Drosophila Activity Monitor and vial plugs were put onto both ends to keep the drosophila medium from drying. The intervals on the Drosophila Activity Monitor were set at two minutes.

Image J Protocols

In order to quantify fluorescence of neurons, measurements was set to area, integrated density, and mean gray value. The cell of interest was selected using the rectangular drawing tool and measured. The measurements produced are the cell count, the total area of the cell, and the average integrated density of the cell. Then three measurements of the background fluorescence were taken by using the drawing tool, outlining three random background areas, and taking the same measurements. The average of the background fluorescence measurements was taken. In order to calculate the corrected total cell fluorescence (CTCF) the formula CTCF=Total Integrated Density of Neurons-(Total Area of Neurons X Mean Fluorescence of Background) was used.

Statistical Analysis

Data was analyzed using the SPSS software. Data for all results were analyzed using a One way ANOVA followed by a post hoc Scheffe test ($p<0.05$).

RESULTS

Body Bend Frequency

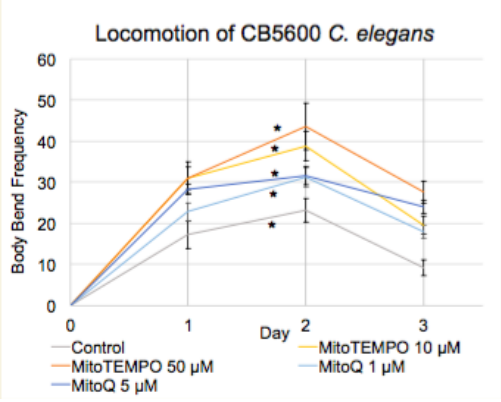

Figure 1. Comparing body bend frequency of untreated control CB5600 *C. elegans* (n=150), with *C. elegans* treated with varying concentrations of the mitochondria-targeted antioxidants MitoTEMPO and MitoQ (n=150) over a period of 3 days. Asterisks represent statistical significance ($p<0.05$).

The x-axis represents the different groups, which included the control, the groups exposed to MitoQ (1 µM and 5 µM), and those exposed to MitoTEMPO (10 µM and 50 µM). The y-axis represents the number of body bends observed in a time interval of one minute.

The untreated worms had a significantly lower body bend frequency as compared to the treated worms. Although MitoTEMPO originally increased the motor functions of the worms by a greater amount than MitoQ, MitoQ created a more gradual decline in motor ability as opposed to a rapid decrease. Overall, the data suggests that MitoQ and MitoTEMPO are effective treatments in targeting the motor disabilities associated with PD in the model organism *C. elegans.*

Chemotaxis Assay

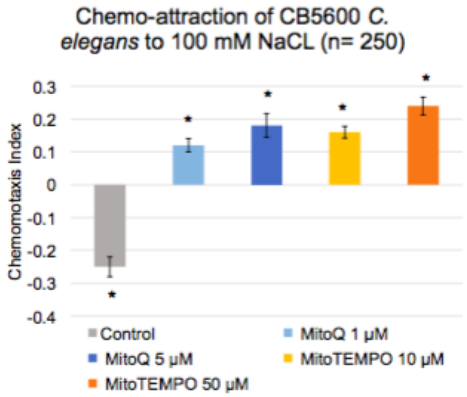

Figure 2. Comparing the chemo-attraction from a chemotaxis assay of control lead nitrate exposed CB5600 *C. elegans*, MitoQ (1 and 5 µM) treated CB5600, and MitoTEMPO (10 and 50 µM) treated CB5600. Standard deviations are shown as error bars. Asterisks represent statistical significance ($p<0.05$).

The x-axis represents the various groups of *C. elegans*: control lead nitrate exposed CB5600 *C. elegans*, MitoQ (1 and 5 µM) treated CB5600, and MitoTEMPO (10 and 50 µM) treated CB5600. The y-axis represents the chemotaxis index measured using 1 mM NaCl as a chemo-attractant. It was found that lead nitrate exposed CB5600 had severely decreased chemoattraction, but both MitoQ and MitoTEMPO significantly increased the olfactory chemo-attraction of *C. elegans*.

Fluorescence

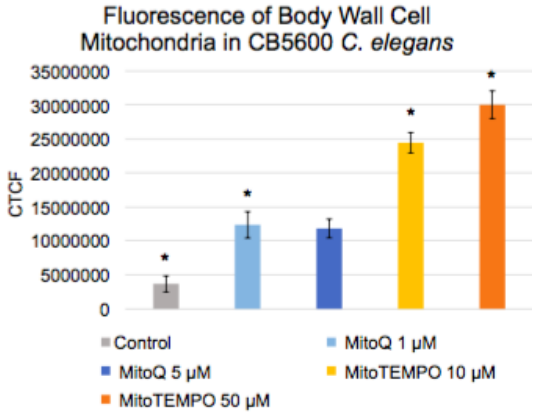

Figure 3. Comparing the quantified fluorescence of body wall cell mitochondria of Lead nitrate exposed and untreated control, MitoQ (1 µM and 5 µM), and MitoTEMPO (10 µM and 50 µM) CB5600 *C. elegans.* Asterisks represent statistical significance ($p<0.05$).

The x-axis represents the different groups of *C. elegans*: the control, MitoQ (1 and 5 µM), and MitoTEMPO (10 and 50 µM) groups of CB5600 *C. elegans*. The y-axis represents the quantified fluorescence as observed through a Zeiss Axiovert fluorescent microscope and analyzed by ImageJ. Figure 3 shows that all MitoTEMPO treated groups exhibited significantly greater fluorescence of mitochondria than the untreated PD worms and MitoQ 1 µM treated nematodes also exhibited significantly greater levels of mitochondria.

Aspect 2 Results

Sleep Behavior Assay

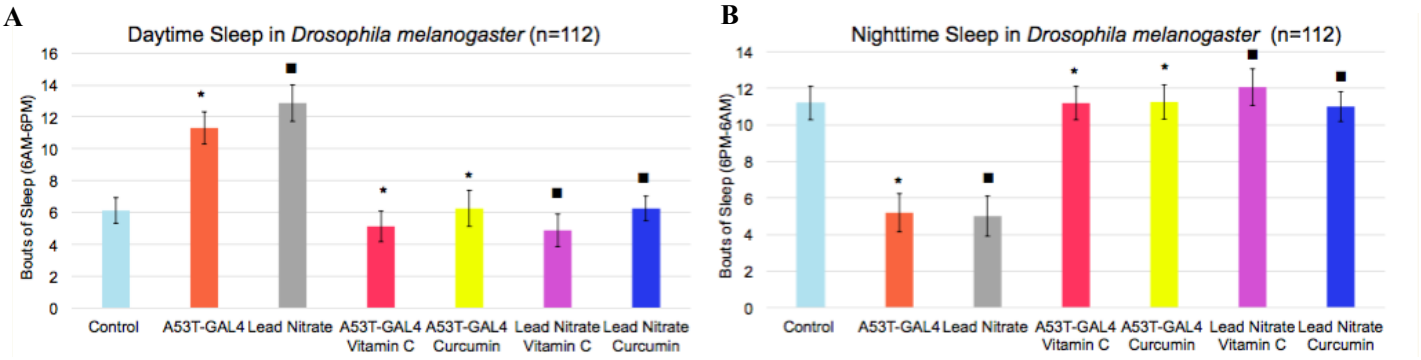

Figure 4. Comparing sleep behavior in number of bouts of sleep of *Drosophila melanogaster* during the **A**. daytime (6AM-6PM) and **B**. nighttime (6PM-6AM). Standard deviation is shown as error bars. Asterisks represent significance with respect to A53T-GAL4 and squares represent significance with respect to lead nitrate exposure Drosophila ($p<0.05$).

The x-axis represents the different groups of Drosophila: the control GFP-GAL4, mutant A53T-GAL4, the Lead nitrate (0.5 mg/mL) exposed GFP-GAL4, Vitamin C (80 µM) treated A53T-GAL4, Curcumin (80 µM) treated A53T-GAL4, Vitamin C treated Lead Nitrate, and Curcumin treated Lead Nitrate *Drosophila melanogaster*. The y-axis represents the number of bouts of sleep, defined by 5 or more minutes of inactivity in the Drosophila Activity Monitor (DAM2). Figure 2A shows the bouts of sleep during the daytime (6AM-6PM) and figure 2B shows the bouts the sleep during the nighttime (6PM-6AM). In figure 4A, it is shown that the Parkinson's disease model flies, A53T-GAL4 and lead nitrate exposed, had a much greater number of bouts of sleep during the daytime compared to the control Drosophila. This excessive inactivity, however, was saved by exposure to Vitamin C and Curcumin. In figure 4B, mutant A53T-GAL4 and lead nitrate exposed Drosophila experienced large reductions in sleep during the nighttime compared to the control group. These sleep bout numbers were brought to normal by exposure to Vitamin C and Curcumin.

Negative Geotaxis Assay

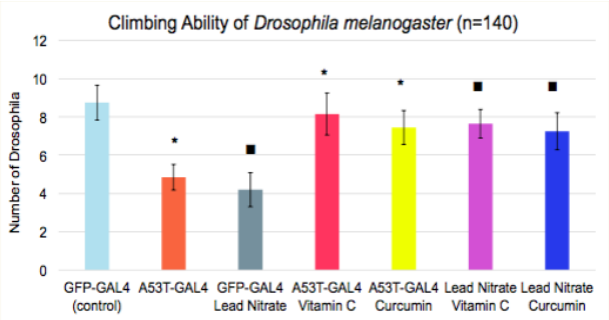

Figure 5. Comparing the climbing abilities from a negative geotaxis assay of *Drosophila melanogaster*. Asterisks represent significance with respect to A53T-GAL4 and squares represent significance with respect to lead nitrate exposure Drosophila ($p<0.05$).

The x-axis represents the different groups of Drosophila: the control GFP-GAL4, mutant A53T-GAL4, the Lead nitrate (0.5 mg/mL) exposed GFP-GAL4, Vitamin C (80 µM) treated A53T-GAL4, Curcumin (80 µM) treated A53T-GAL4, Vitamin C treated Lead Nitrate, and Curcumin treated Lead Nitrate *Drosophila melanogaster*. The y-axis represents the average number of Drosophila that passed the 8 cm line on the Drosophila negative geotaxis chamber in 10 seconds. The Drosophila models of Parkinson's disease, mutant A53T-GAL4 and Lead nitrate exposed GFP-GAL4 Drosophila, had less Drosophila pass the 8 cm line than the control Drosophila. This indicates that aggregated alpha-synuclein and exposure to lead nitrate decreases locomotor ability in *Drosophila melanogaster*. However, this degradation in climbing ability was saved by Vitamin C and Curcumin treatment, suggesting that Vitamin C and Curcumin are effective in alleviating decreased locomotion in Parkinson's disease.

Fluorescence

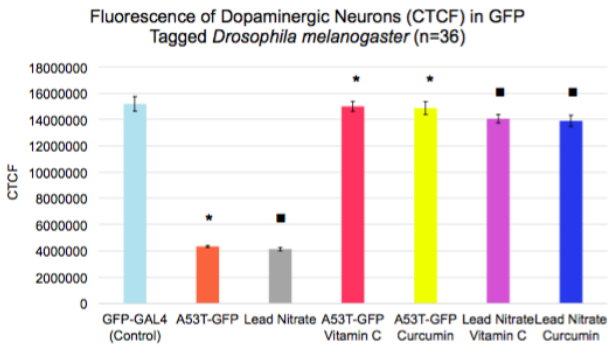

Figure 6. Comparing the fluorescence of dopaminergic neurons of *Drosophila melanogaster*. Standard deviation is shown as error bars. Asterisks represent significance with respect to A53T-GFP and squares represent significance with respect to lead nitrate exposure Drosophila ($p<0.05$).

The x-axis represents the different groups of Drosophila: the control GFP-GAL4, mutant A53T-GAL4, the Lead nitrate (0.5 mg/mL) exposed GFP-GAL4, Vitamin C (80 µM) treated A53T-GAL4, Curcumin (80 µM) treated A53T-GAL4, Vitamin C treated Lead Nitrate, and Curcumin treated Lead Nitrate *Drosophila melanogaster*. The y-axis represents the fluorescence of the dopaminergic neurons of the Drosophila. It was found that the dopaminergic neuron fluorescence of the Drosophila models of Parkinson's disease (Lead nitrate exposed and A53T-GFP) were significantly decreased, but the fluorescence was significantly increased through Vitamin C and Curcumin treatment.

DISCUSSION

The purpose of this study was to test the effectiveness of MitoQ, MitoTEMPO, Curcumin, and Vitamin C in ameliorating the Parkinsonian symptoms in *C. elegans* and *Drosophila melanogaster*.

In aspect 1, it was found that lead nitrate lead to decreased motor function in CB5600 *C. elegans*, a symptom of parkinsonism. Lead nitrate, a heavy metal, depletes the major antioxidants in cells, leading to increased ROS levels and oxidative stress. This, in turn, puts strain on mitochondria, which dopaminergic neurons rely heavily on, ultimately leading to DAergic neurodegeneration. The ROS-associated motor deficits observed are supported by the results of Patra (2011) because the redox-inactive metal, lead nitrate, increased oxidative stress [18]. In addition, it was found that lead nitrate, due to the strain on mitochondria from increased ROS levels, also decreased the fluorescence of body wall cell mitochondria.

Upon treating the worms with the mitochondria-targeted antioxidants, it was found that the antioxidants decreased the detrimental effects of PD-related oxidative stress on locomotion, as the worms treated with ether MitoQ or mitoTEMPO exhibited an increase in the number of body bends per minute as compared to the untreated control.

It was also found that both MitoQ and mitoTEMPO significantly ameliorated the decreased chemoattraction of lead nitrate exposed *C. elegans*. It was also found that *C. elegans* treated with the mitochondria-targeted antioxidants had recovered levels of of mitochondria in the body wall muscle cells when compared to the decrease in that of the control. The quantified fluorescence was observably greater in both MitoQ and MitoTEMPO groups, with MitoTEMPO having the greatest ameliorating effects. These results are supported by those of Sheu (2006) because antioxidants lower levels of oxidative stress which, when high, can damage mitochondrial DNA and contribute to mitochondrial dysfunction [25].

In aspect 2, it was found that alpha synuclein aggregation causes Parkinsonian symptoms in Drosophila melanogaster. The A53T-GAL4 Drosophila that exhibited aggregated alpha synuclein in the dopaminergic neurons experienced a shift in their circadian rhythm, with them being less active during the daytime than control flies, and a greatly decreased climbing ability than the control flies. These results are supported by those of Jackle (2011) because the Drosophila that exhibited the prefibrillar alpha synuclein oligomers experienced excessive daytime sleepiness [4]. This is because the oligomers disrupt the neuronal system by blocking the transport of dopamine [13]. These results also suggest that dopaminergic neurons may have a role in maintaining circadian rhythm in Drosophila melanogaster.

It was also found that the Drosophila exposed to lead nitrate experienced abnormal sleep behavior and decreased mobility, which is supported by the results of Neal (2012) [17]. Because lead nitrate has been shown to induce oxidative stress by increasing ROS generation by over active microglial cells, it is likely that the oxidative stress caused the death of dopaminergic neurons, and thus the disruption of the transport of dopamine (Patra, 2011) [18]. Because dopamine is very important in the motor function of organisms, decreased dopaminergic neuron functioning would cause a decrease in the mobility of the Drosophila (Jankovic, 2007) [3].

It was found that Vitamin C ameliorated the mobility dysfunction of the Drosophila that exhibited aggregated alpha synuclein in their dopaminergic neurons and were exposed to lead nitrate. This is supported by the results of Khan (2012) because the Vitamin C greatly increased the climbing ability of the Drosophila model of Parkinson's disease [22]. Vitamin C was also able to cause the sleep behavior of alpha synuclein mutant Drosophila to return to the same as the control Drosophila sleep behavior. This could be explained by the fact that Vitamin C is an antioxidant and can protect against oxidative stress in dopaminergic neurons. This protection against oxidative stress can decrease degeneration of dopaminergic neurons and increase synaptic transmission of dopaminergic neurons [26].

Curcumin was also found to have increased climbing abilities and ameliorated the excessive sleepiness of Drosophila affected with Parkinson's disease through alpha synuclein aggregation and lead nitrate exposure. These findings were supported by the studies of Phom

(2014) because Curcumin increased the climbing ability of Parkinson's disease affected Drosophila [21]. Curcumin was also able to decrease the excessive sleepiness of the Parkinson's disease affected Drosophila during the daytime. This can be explained by how the antioxidant nature of Curcumin can remodel alpha synuclein aggregates into non-toxic fibrils and thus can decrease ROS overproduction [27].

The limitation of this study was that an in vitro study could not be performed, so the ROS levels could not be quantified exactly in the model organisms.

CONCLUSION

The results from aspect 1 demonstrate that lead nitrate effectively increases cellular oxidative stress and therefore induces mitochondrial dysfunction and neurodegeneration, indicated by the decrease in motor ability of *C. elegans*. This suggests that lead nitrate can be used to induce parkinsonism and stimulate complex malfunction in the model organism *C. elegans* strain CB5600. It was also found the MitoQ is effective in both increasing the locomotion and decreasing the decline of motor function over time. MitoQ was also found to be effective in increasing the presence of mitochondria in the *C. elegans*. MitoTEMPO was found to be an effective agent in increasing locomotion capacity, although it failed to address the gradual decline in locomotion of the worms. In addition, MitoTEMPO was the most effective in increasing the number of mitochondria, a necessary component of cellular function, especially in dopaminergic neurons, present in the worms.

The results from aspect 2 showed that aggregated α-synuclein negatively affects both sleep behavior and locomotion in Drosophila melanogaster. It was also found that lead nitrate causes Parkinsonian symptoms in Drosophila melanogaster similar to those of aggregated α-synuclein, with a decreased ability in locomotion and abnormal increased sleep during the daytime. Vitamin C was successfully able to increase the locomotion and decrease the daytime sleepiness of the Drosophila with aggregated α-synuclein in the dopaminergic neurons, which suggests that Vitamin C can be helpful in ameliorating the locomotor deficiencies and abnormal that are characteristic of Parkinson's disease. Vitamin C was also effective in ameliorating the locomotor deficiencies and abnormal sleep of Drosophila exposed to lead nitrate. Curcumin increased locomotor abilities and decreased excessive sleepiness during the daytime in both Drosophila with aggregated α-synuclein in the dopaminergic neurons and exposure to lead nitrate, suggesting that Curcumin is also an effective antioxidant in combatting the overproduction of ROS and subsequent oxidative stress.

FUTURE STUDIES

To further investigate the Parkinson's disease pathogenesis, the pathways affected by the genetic mutations DJ-1, LRRK2, and UCH L1 can be investigated. DJ-1 has been speculated to prevent the correction of misfolded α-synuclein, which can lead to mitochondrial dysfunction [28]. Mutations in the LRRK2 gene blocks excess α-synuclein from being degraded, which can cause the development of Lewy bodies [29]. UCH L1 provides the instructions to make the ubiquitin enzyme, which tags damaged proteins for degradation, so a mutation in this gene could cause the aggregation of α-synuclein into Lewy bodies. Furthermore, the effect of antioxidants on lifespan could be studied and the levels of cellular ROS could also be measured.

ACKNOWLEDGEMENTS

We would like to thank Ms. Alison Huenger and Mrs. Virginia Flanagan for their unwavering support and guidance. Their insight and expertise greatly aided in our research.

REFERENCES

1. "Parkinson's Disease Foundation (PDF)." *What Is Parkinson's Disease? | Parkinson's Disease Foundation (PDF)*. N.p., n.d. Web. 16 June 2016.

2. "Parkinson's Disease (PD)." *Florida Hospital*. N.p., n.d. Web. 27 Apr. 2016.
3. Jankovic, J. "Parkinson's Disease: Clinical Features and Diagnosis." *Journal of Neurology, Neurosurgery & Psychiatry*. BMJ Publishing Group Ltd, 01 Apr. 2008. Web.
4. Jäckle, Herbert. "Pre-Fibrillar α-Synuclein Mutants Cause Parkinson's Disease-Like Non-Motor Symptoms in Drosophila." *Journals.plos.org*. N.p., 8 Sept. 2011. Web. 12 June 2016.
5. Lee, Virginia M.-Y. "Pathological α-Synuclein Transmission Initiates Parkinson-like Neurodegeneration in Non-transgenic Mice." *Ncbi.nlm.nih.gov*. U.S. National Library of Medicine, 16 Nov. 2012. Web. 04 May 2016.
6. Pifl, Christian. "Is Parkinson's Disease a Vesicular Dopamine Storage Disorder? Evidence from a Study in Isolated Synaptic Vesicles of Human and Nonhuman Primate Striatum." *Jneurosci.org*. N.p., 11 June 2014. Web. 4 May 2016.
7. Mason, Jody. "Intracellular Screening of a Peptide Library to Derive a Potent Peptide Inhibitor of α-synuclein Aggregation." *Jbc.org*. N.p., 23 Jan. 2015. Web. 04 May 2016.
8. Greenamyre, Timothy. "Complex I and Parkinson's Disease." *Onlinelibrary.wiley.com*. N.p., 2001. Web. 4 May 2016.
9. Deierborg, Tomas. "Microglia-Secreted Galectin-3 Acts as a Toll-like Receptor 4 Ligand and Contributes to Microglial Activation." *Cell.com*. N.p., 10 Mar. 2015. Web. 4 May 2016.
10. Espay, Alberto. "Parkinson's Disease (PD)." *Mayfield Clinic*. N.p., Apr. 2016. Web. 05 June 2016.
11. Lull, Melinda E., and Michelle L. Block. "Microglial Activation & Chronic Neurodegeneration." *Neurotherapeutics: The Journal of the American Society for Experimental NeuroTherapeutics*. U.S. National Library of Medicine, 05 Oct. 2010. Web. 05 June 2016.
12. Herczenik, E., and M. F. B. G. Gebbink. "Molecular and Cellular Aspects of Protein Misfolding and Disease." *The FASEB Journal* 22.7 (2008): 2115-133. Web. 5 June 2016.
13. Harrington, Adam J., Shusei Hamamichi, Guy A. Caldwell, and Kim A. Caldwell. "C. Elegans as a Model Organism to Investigate Molecular Pathways Involved with Parkinson's Disease." *Developmental Dynamics* (2010): n. pag. Web. 2 May 2016.
14. Murphy, Michael P. "How Mitochondria Produce Reactive Oxygen Species." *Biochemical Journal* 417.Pt 1 (2008): 1-13. *Biochemical Journal*. Portland Press Ltd., 01 Jan. 2009. Web. 4 May 2016.
15. Keane, P. C., M. Kurzawa, P. G. Blaine, and C. M. Morris. "Mitochondrial Dysfunction in Parkinson's Disease." *Mitochondrial Dysfunction in Parkinson's Disease,* 2011 (2011): n. pag. *Parkinson's Disease*. Hindawi Publishing Corporation., Web. 18 May 2016.
16. Jin, Huajun. "Mitochondria-targeted Antioxidants for Treatment of Parkinson's Disease: Preclinical and Clinical Outcomes." *Sciencedirect.com*. N.p., 20 Sept. 2013. Web. 02 June 2016.
17. Neal, April. "Mechanisms of Heavy Metal Neurotoxicity: Lead and Manganese." *J Drug MetabToxicol* 42.10-11 (2013): 686. *Omicsonline.org*. 14 Apr. 2013. Web. 4 May 2016.
18. Patra, R. C., Amiya K. Rautray, and D. Swarup. "Oxidative Stress in Lead and Cadmium Toxicity and Its Amelioration." *Veterinary Medicine International* 2011 (2011): n. pag. SAGE-Hindawi Access to Research, 2011. Web. 17 May 2016.
19. Ng, Li Fang, Jan Gruber, Irwin K. Cheah, Chong Kit Goo, Wei Fun Cheong, Guangho Shui, Kim Ping Sit, Marcus R. Wenk, and Barry Halliwell. "The Mitochondria-targeted Antioxidant MitoQ Extends Lifespan and Improves Healthspan of a Transgenic Caenorhabditis Elegans Model of Alzheimer Disease." *Free Radical Biology and Medicine* 71 (2014):390-401. Print.

20. Johnson, D., E. Allman, and K. Nehrke. "Regulation of Acid-base Transporters by Reactive Oxygen Species following Mitochondrial Fragmentation." *AJP: Cell Physiology* 302.7 (2012): n. pag. Web. 23 Apr. 2016.
21. Phom, Limamanen."Curcumin's Neuroprotective Efficacy in *Drosophila* Model of Idiopathic Parkinson's Disease Is Phase Specific: Implication of Its Therapeutic Effectiveness." *Rejuvenation Research*. Mary Ann Liebert, Inc., 01 Dec. 2014. Web. 01 June 2016.
22. Khan, Safiya. "Effect of L-Ascorbic Acid on the Climbing Ability and Protein Levels in the Brain Of Drosophila Model of Parkinson's Disease." *International Journal of Neuroscience* 122.12 (2012): 704-09. Web.
23. Iyer, Eswar Prasad R., Srividya Chandramouli Iyer, and Daniel N. Cox. "Application of Cell-Specific Isolation to the Study of Dopamine Signaling in *Drosophila*." *Methods in Molecular Biology (Clifton, N.J.)*. U.S. National Library of Medicine, 30 Oct. 2014. Web. 06 June 2016.
24. Williamson, W. Ryan, and P. Robin Hiesinger. "Preparation of Developing and Adult *Drosophila* Brains and Retinae for Live Imaging." *Journal of Visualized Experiment : JoVE*. MyJove Corporation, 2010. Web.
25. Sheu, Shey-Shing. "Targeting Antioxidants to Mitochondria: A New Therapeutic Direction." *Biochimica Et Biophysica Acta (BBA) –Molecular Basis of Disease* 1762.2 (2006): 256-65. *Targeting Antioxidants to Mitochondria: A New Therapeutic Direction*. Web. 20 May 2016.
26. Siddique, Yasir Hasan. "Effect of Curcumin on Lifespan, Activity Pattern, Oxidative Stress, and Apoptosis in the Brains of Transgenic Drosophila Model of Parkinson's Disease." *Effect of Curcumin on Lifespan, Activity Pattern, Oxidative Stress, and Apoptosis in the Brains of Transgenic Drosophila Model of Parkinson's Disease*. N.p., 17 Apr. 2014. Web.
27. Sgarbossa, Antonella. "Natural Biomolecules and Protein Aggregation: Emerging Strategies against Amyloidogenesis." *International Journal of Molecular Sciences* 13.12 (2012): 17121-7137. Web. 24 May 2016.
28. Zhang, Wei. "Aggregated α-synuclein Activates Microglia: A Process Leading to Disease Progression in Parkinson's Disease." *Fasebj.com*. N.p., Apr. 2005. Web. 06 June 2016.
29. Di Fonzo, Alessio. "A frequent *LRRK2* gene mutation associated with autosomal dominant Parkinson's disease." *The Lancet* 365.9457 (2005): 412-415. Web. 03 June 2016