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The Use of Curcumin to Mitigate the Inclusion of α-synuclein in Transgenic *Caenorhabditis elegans*

Abstract

Aggregation of the protein α-synuclein causes symptoms of Parkinson's Disease in human brains. Treatment of transgenic NL5901 *C. elegans* with the xenohormetic phytochemical curcumin was administered to decrease the aggregation of α-synuclein and maintain the proteostasis of the nematode. NGM agar plates with and without 20μM curcumin were seeded with OP50 *E. coli*, and worms were then transferred onto the plates and allowed to grow to adulthood. Viewing of YFP-tagged α-synuclein occurred via Carl Zeiss Axiovert 40 CFL fluorescence microscope at 514 nm, and images were analyzed for fluorescence intensity via ImageJ software. Fluorescence intensity of treated worms was observed to be significantly ($p<0.05$) lower than untreated worms, suggesting that curcumin has the ability to decrease alpha synuclein aggregation in aging animals.

Introduction

Parkinson's Disease is a neurodegenerative disorder in which the aggregation of α-synuclein and the substantial loss of dopaminergic neurons in the substantia nigra causes a wide array of symptoms. These symptoms include, but are not limited to rigidity, tremors, and bradykinesia (DeMaagd *et. al*, 2015). As a result of the pathogenesis of Parkinson's, patients lose the ability to perform Activities of Daily Living (ADL), which significantly decreases both the independence and the quality of life of affected individuals (QoL). This decrease in QoL and inability to perform ADL affects not only the life of the patient, but also the lives of the families of diseased individuals, causing a financial and emotional strain on the entire family. As the life expectancy of the population increases, incidents of neurodegenerative disease are projected to increase proportionally, making the treatment of such diseases a global health concern (Oliveira de Carvalho *et. al*, 2018).

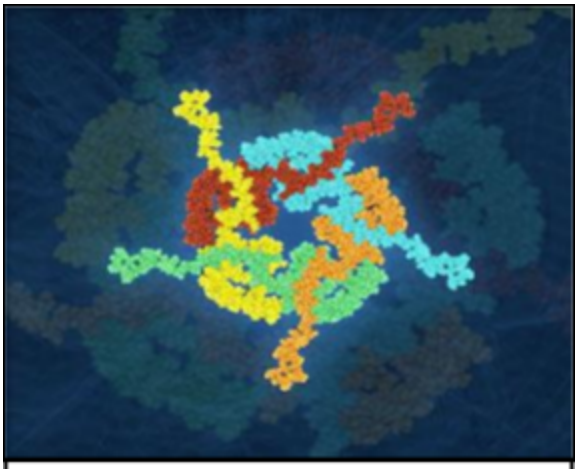

Fig.1 The molecular structure of alpha synuclein, the protein whose misfolding is closely associated with the development of Parkinson's Disease. Here, alpha synuclein is properly folded and therefore soluble. When alpha synuclein misfolds into β- sheet inclusions, it forms Lewy Bodies. These inclusions are insoluble, and build up in the brains of Parkinson's Disease, causing the death of neurons (*Image via michaeljfox.org*).

Parkinson's Disease can be caused by several factors, including genetic mutations in genes such as SNCA, the gene that codes for alpha synuclein production, and LRRK2, which codes a Leucine Rich Repeat Kinase (Cooper *et. al*, 2015). A key pathological hallmark of Parkinson's Disease is the misfolding of the protein α-synuclein into fibrils and oligomers (Rockenstein *et. al*, 2014). When properly folded, α-synuclein is shaped helically and localizes in the presynaptic terminals of nerves (Fig 1) (Bendor *et. al*, 2013). However, in the brains of Parkinson's patients the misfolded α-synuclein aggregates into β- sheet inclusions to form the fibrous Lewy Bodies (Bodicharla *et. al*, 2012). This inclusion of misfolded α-synuclein increases with age and the associated progressive neurodegeneration, especially in that the inclusions are made from increasingly election-dense and granular misfolded proteins (van Ham *et. al*, 2008). The misfolded, granular form of α-synuclein is insoluble in the cell, which allows the protein to accumulate inside the cells into the aforementioned inclusions, causing a disruption in the protein homeostasis of an individual's neurons (Moronetti *et. al*, 2012). This offsetting of the protein homeostasis of the individual's neurons can be a result of oxidative

stress in the Endoplasmic Reticulum, which synthesizes proteins destined for cell membranes such as α-synuclein (Tsai *et. al*, 2010).

In a healthy brain, misfolded proteins are cleared out by the Unfolded Protein Response (UPR), which is triggered by increased levels of intracellular ROS in response to the toxicity of the proteins (Kozlowski *et. al*, 2014). The toxicity of proteins not only induces the formation of intracellular ROS, but also disrupts cellular protein homeostasis, which is maintained via the alteration of the cellular folding environment in order to meet folding demands caused by physiological or environmental stressors (Moronetti *et. al*, 2012). The UPR clears out the misfolded proteins through the activation of genes that support cellular proteostasis and restore ER function. The molecular pathway of the Unfolded Protein Response, from the disruption of proteostasis to the signalling for gene upregulation to support ER stress, is outlined in Figure 2. Examples of these Unfolded Protein Response genes include genes which induce pathways to increase the folding capacity of the ER, as well as genes that upregulate the production of lysosomes and autophagosomes, effectively increasing the rate of autophagy in cells (Tsai *et. al*, 2010). This gene activation results in autophagy, which envelops the misfolded proteins in autophagosomes, and fuses the autophagosome with a lysosome to degrade the toxic material (Zhang *et. al*, 2018). However, in the brain of a Parkinson's patient, the rate of production of toxic misfolded proteins exceeds the rate of autophagy via the UPR, which allows the proteins to form the aforementioned inclusions.

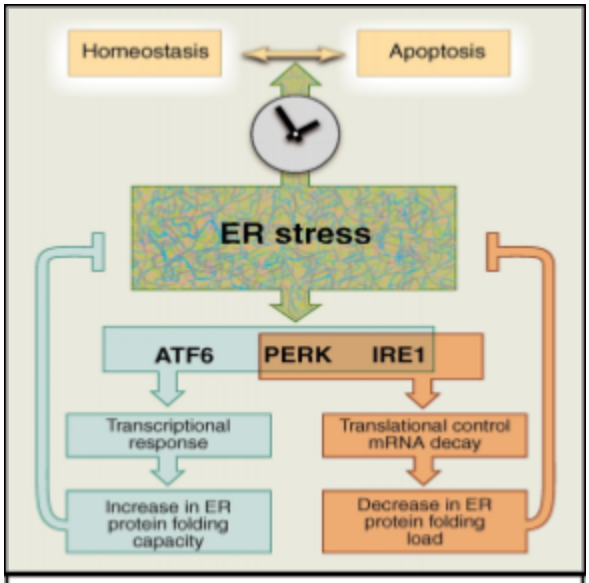

Fig. 2 The molecular pathway of the Unfolded Protein Response, which is triggered by a disruption in the protein homeostasis of an individual. Disruption in proteostasis leads to an increase in Endoplasmic Reticulum stress, which leads to the alterations in gene expression mentioned above (*Image via science.sciencemag.org*).

Another method of inducing autophagy is through the evocation of the hormetic response, a cytoprotective stress response to toxic chemicals produced outside of the cell (Zhang *et. al*, 2018). Hormetic responses are induced by xenohormetic phytochemicals, which are chemicals produced by

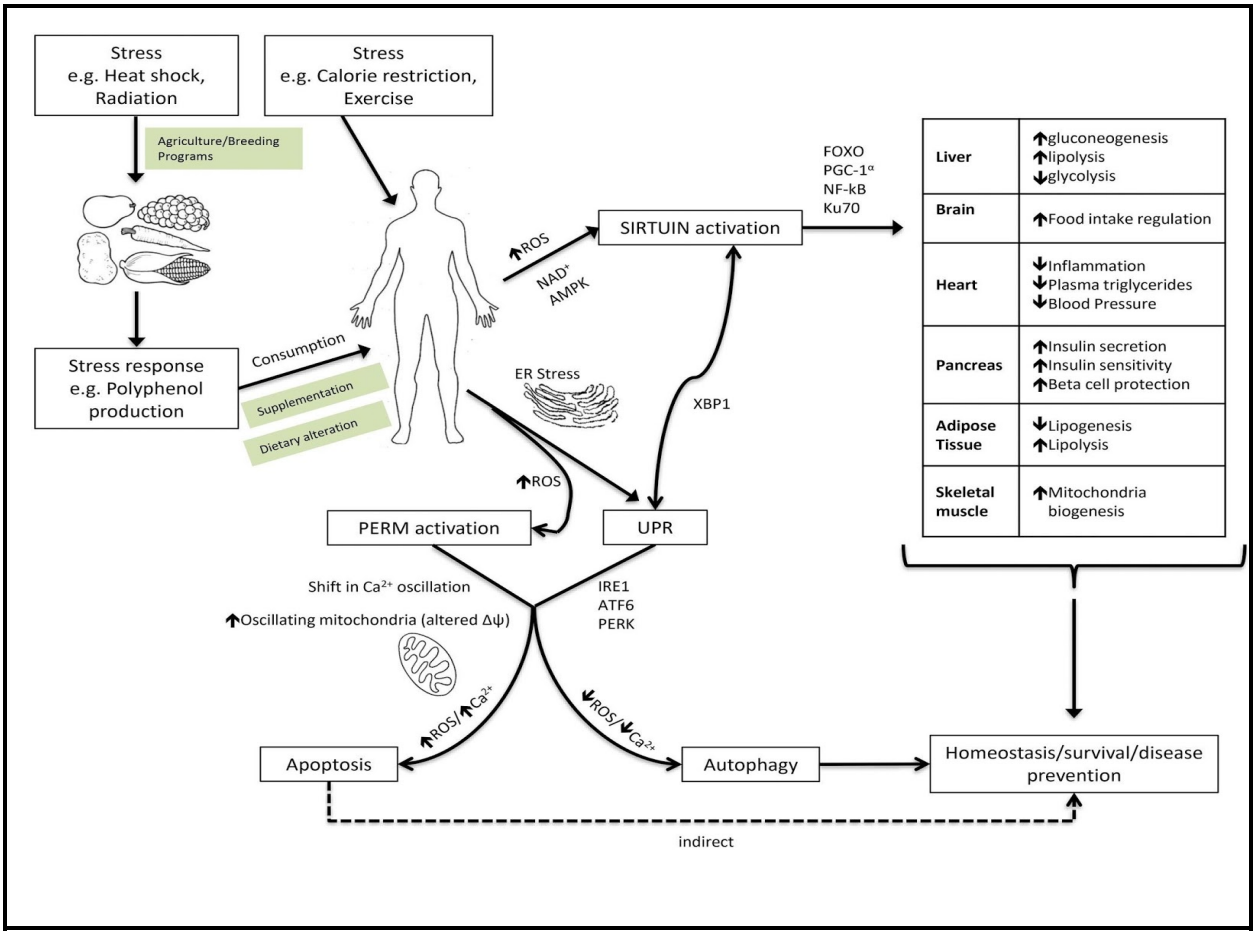

Fig. 3 The process of xenohormesis, which is the conferral of stress response benefits from plant stress products to human beings. Plant stress response products, such as curcumin, are toxic to humans, and elicit a biphasic hormetic response that is known to promote human health. These health benefits include the increase in stress tolerance, and the slowing of the aging process. *Image via (Suter et. al 2017)*

plants in times of physiological stress (Maulik *et. al*, 2018). This is evident due to the fact that the phytochemicals are toxic to human cells and elicit the biphasic response pattern attributed to hormesis through the causation of organelle damage and elevated levels of intracellular ROS (Fang *et. al*, 2017). The hormetic responses are dose-dependent; too small a dose fails to elicit a response while too large a dose will kill the cell via the triggering of apoptosis (Srivastava *et. al*, 2008). The triggering of apoptosis during the hormetic response occurs due to the presence of organelle damage beyond possible repair, and levels of intracellular ROS far beyond the autophagic threshold in response to the introduced toxic substances (Maulik *et. al*, 2018).

Curcumin is a xenohormetic phytochemical derived from the plant *Curcumin longa* (Liao *et. al*, 2011). The molecular structure of curcumin, including its polyphenolic rings, is shown in Figure 4. Due to its being a xenohormetic chemical, it induces a biphasic hormetic response in animal cells, which leads

to elevated levels of intracellular ROS and the triggering of autophagy. Curcumin triggers autophagy due to the presence of intracellular ROS, as the cell recognizes the intracellular ROS as toxic and signals for the production of autophagosomes and lysosomes. This signalling occurs via the suppression of the mTOR pathway and the upregulation of TFEB, which together lead to the increased production of lysosomes and the promotion of autophagic events (Zhang *et. al*, 2016).

Parkinson's Disease is the most common synucleinopathy, which is a disease caused by the aggregation of the protein alpha synuclein. Interference with α-synuclein aggregation has been proposed as a viable treatment method for such neurodegenerative diseases, but the complex and disorderly structure of α-synuclein has made structure-based drug design highly difficult (Pujols *et. al*, 2018). Several studies have developed more novel compound-screening methods that have facilitated the discovery of structure-based drugs, however, other methods of interruption of α-synuclein aggregation should be explored to decrease dopaminergic neuron death and to halt the pathogenesis of PD. Therefore, through increasing the phenomena of autophagy in animal cells, an alternate method of halting α-synuclein aggregation is being explored.

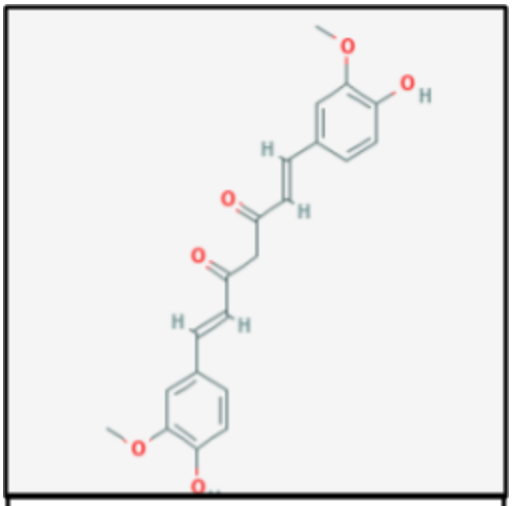

Fig. 4 The molecular structure of curcumin. Curcumin is a derived from the stress response of the *Curcumin longa* plant. It is a polyphenol, as is evident in the two phenolic rings on either end of the molecule. It is an antioxidant, like many polyphenols are, and is being tested for its viability as a treatment for Parkinson's disease. *Image via pubchem.org*

Methods

C. elegans strains and maintenance

NL5901 (nematodes that display YFP tagged alpha synuclein aggregates in body wall muscle cells) *Caenorhabditis elegans* were obtained from the Caenorhabditis Genetics Center (CGC) at the University of Minnesota. All NL5901 *C. elegans* were maintained according to standard protocol on NGM agar with *E. coli* OP50 at 22ºC.

Preparation of curcumin plates

0.7276 grams of curcumin powder were dissolved in 85 ml dimethyl sulfoxide and 50 ml dH_20 to create a stock solution with a molarity of 148.148 µM. 10 ml of the prepared stock solution were then added to 87 ml of dimethyl sulfoxide to create a solution of a 20 µM molarity. 26 ml of the prepared 20 µM solution were added to 174 ml of molten NGM agar and poured into 60 cm dishes for curcumin assays.

Nematode Growth Medium agar preparation

1 ml of 1 M $CaCl_2$, 1 m; of 1 M $MgSO_4$, and 25 ml of 1 M KPO_4 buffer were autoclaved separately, each for 15 minutes. A solution of 3 g of NaCl, 17 g of agar, 2.5 g of peptone, and 975 ml of H_2O was autoclaved for 50 minutes. The solution was left to cool to 55ºC, then mixed with the autoclaved $CaCl_2$, $MgSO_4$, and KPO_4 buffer and 1 ml of 5 mg/ml cholesterol dissolved in ethanol. Petri dishes were then filled up to ⅔ of their volume with the NGM agar and, once solidified, left to sit at room temperature for 1 day. Petri dishes were stored in a refrigerator until seeding procedures were carried out.

Seeding of plates with OP50 E. coli bacteria lawn

A streak of OP50 *E. coli* was prepared from a glycerol stock by selecting one colony to grow in LB overnight at 37ºC with agitation to ensure proper aeration. Once the streak was ready for plate seeding, 100 µl OP50 *E. coli* was placed in the center of the plate with a pipette. A glass tube with a flat hook was then dipped in ethanol and flamed for sterilization purposes, and used to spread the *E.coli* on the NGM plates. The seeded plates were then left to grow overnight in an incufridge before the worms were added to the plates.

Preparation of bleaching solution

2.75 ml of water, 1.23 ml of 1 M sodium hydroxide, and 1 ml of 4% sodium hypochlorite were mixed to produce the bleaching solution. Preparation occurred immediately before age-synchronization bleaching procedure was carried out.

Age synchronization

Four plates of *C. elegans* were washed with 1 ml of M9 buffer two separate times and placed in a centrifuge tube. The eight centrifuge tubes were spun at 3,884 × g (rcf; 8,500 rpm) for 1 min in order to pellet the worms. The resulting supernatant was then discarded. 200 µl of Distilled Water (D.W.) were added to each of the eight centrifuge tubes, and then four of the centrifuge tubes were combined into each of two larger test tubes. 3.2 ml of bleaching solution was added to each of the two test tubes, and the tubes were then shaken until about 90% of nematodes had lysed and several embryos were released, which was around 20 minutes. The test tubes were centrifuged at 3,884 × g (rcf; 8,500 rpm) for 4 minutes to pellet the worms, and the supernatant was discarded without disrupting the pellet for both test tubes. M9 buffer wash was then performed on each test tube, which involved the addition of 1 ml of M9 buffer to each test tube, a subsequent 1 minute centrifuge spin at 137 × g (rcf; 1,000 rpm), and then discarding the supernatant. Two more M9 buffer washes were performed on each test tube, effectively halting the bleaching process. The embryos in both test tubes were then transferred via 1 ml of M9 buffer to a test tube containing 1 ml of M9 (total volume of 2 ml M9 buffer). The single test tube was shaken overnight in a 20 °C water bath with shaking at 30 rpm to allow L1 larvae to hatch. The next day, 100 ml from the test tube was transferred onto each of the 60cm OP50 *E. coli*-seeded NGM agar petri dishes via micropipette.

Curcumin screening

Age synchronized worms at the L1 larva stage were transferred to the prepared curcumin assay petri dishes via subculturing one day after the initial NGM plating. Worms were fed OP50 *E. coli*, and kept at 22°C with the control group petri dishes.

Fluorescence microscope imaging

Worms were picked via micropipette and suspended in a drop of M9 buffer. Worms were transferred via drop of M9 buffer on glass microscope slides for viewing. When M9 buffer evaporated, photos of the worms were taken under the Carl Zeiss Axiovert 40 CFL fluorescence microscope via Canon camera at 1X magnification. This process occurred on both days 6 and 8 of adulthood.

ImageJ analysis of images

ImageJ software was downloaded via imagej.nih.gov. The images were opened in ImageJ, and the freehand selection tool was used to outline the bodies of worms. From the Analyze menu, "Set Measurements" was selected. The "Integrated Density" button was selected for calculation, to determine the level of aggregation of α-synuclein::YFP in the *C. elegans*. Integrated Density was selected to represent this quantity due to the amount of fluorescence of the YFP correlating directly with the density of green in the worm images. Then, from the Analyze menu, "Measure" was selected and the measurements were obtained for each worm. Integrated Density was used as the sole indicator of fluorescence intensity, and unpaired T-tests were performed between the Adult Day 6 groups and the Adult Day 8 groups respectively. P-values of less than 5% were accepted as statistically significant.

Results

Curcumin Treatment decreases alpha synuclein aggregation in day 6 worms

ImageJ analysis of 60 photos captured of adult day 6 worms showed a significant ($p < 0.05$) decrease in fluorescence intensity in curcumin treated groups (Figure 5). Mean integrated density of treated worms on day 6 was 8830961.13 ± 3264944 for 30 samples. Untreated worms displayed a mean integrated density of 29583706.17 ± 17893621 for 30 samples (Figure 6). Treatment of worms decreased the mean fluorescence intensity of day 6 adults by ~29.9% ($p < .05$).

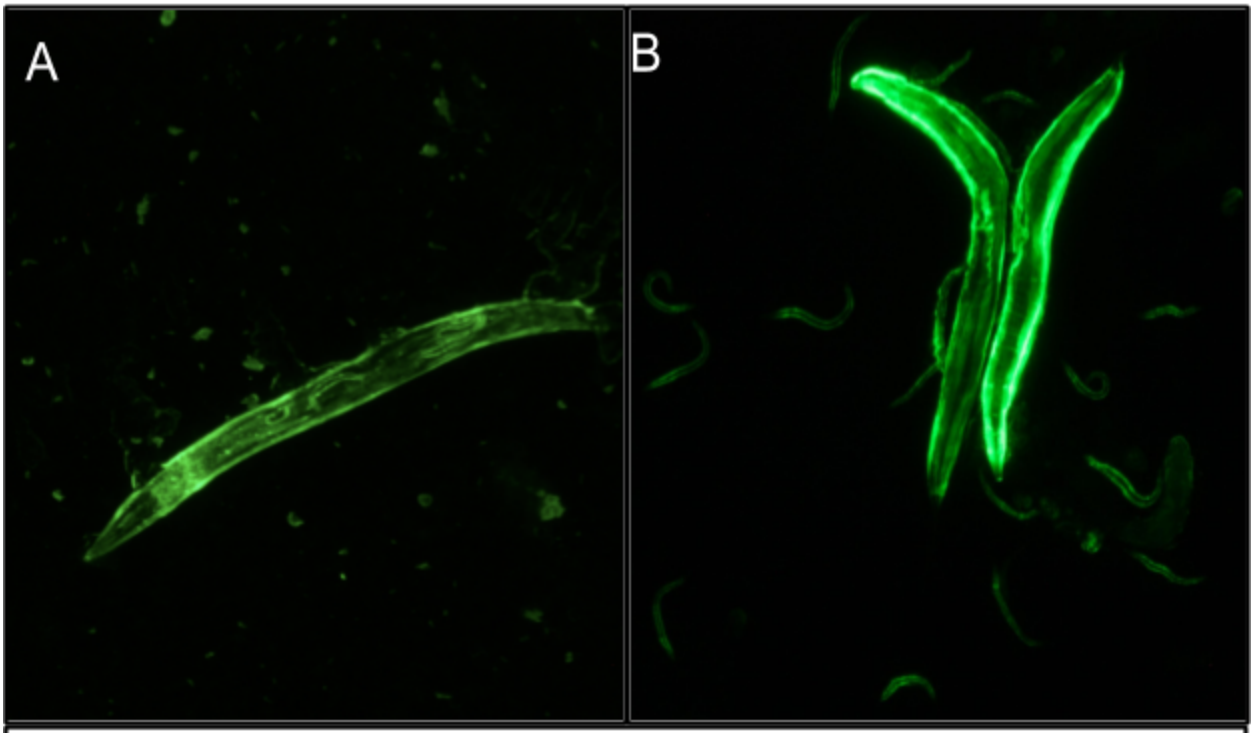

Fig. 5 Images of Adult Day 6 age synchronized NL5901 *C. elegans*. (A) Grown on NGM agar plates seeded with curcumin (20 μM) and (B) worms grown on NGM agar plates without curcumin seeding. ImageJ's Integrated Density measurement was used to measure level of aggregation, through outlining worms with the freehand selection tool. Fluorescence intensity was determined using the Integrated Density values for each of 30 worm samples in both groups A and B. T-test analysis was conducted to determine that treated worms displayed significantly ($p < .05$) less alpha synuclein aggregation than their untreated counterparts (Created by student researcher).

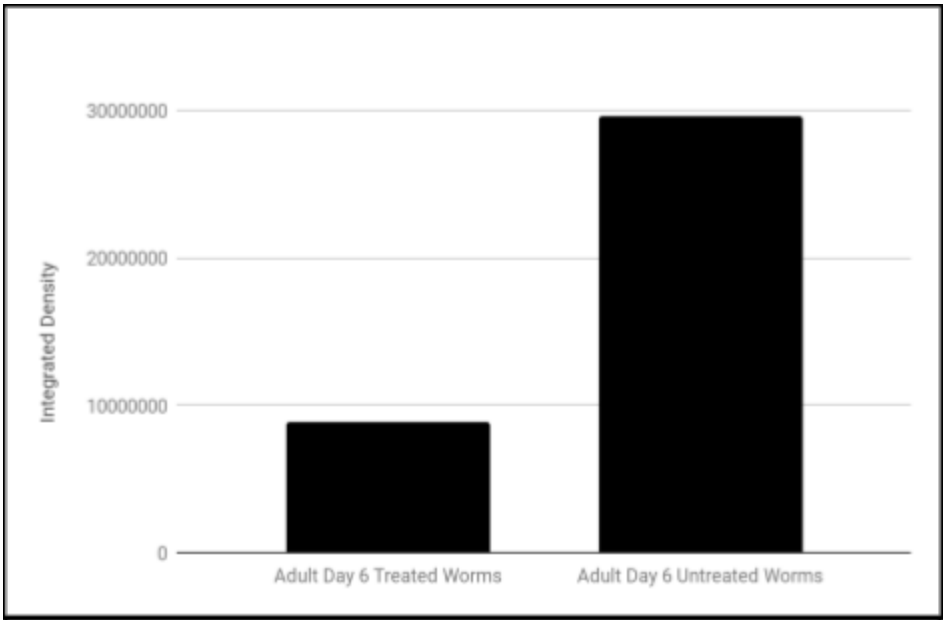

Fig. 6 Mean Integrated Density measurements for age synchronized Adult Day 6 NL5901 worms. The Integrated Density of Treated worms, grown on curcumin-seeded NGM agar plates (concentration = 20 μM), is significantly ($p<.05$) lower than the Integrated Density of Untreated worms, grown on unseeded NGM agar plates. This suggests less fluorescence intensity in treated worms, which correlates with lower levels of alpha synuclein aggregation. Integrated Density measurements taken via ImageJ's Integrated Density measurement after using the freehand tool to outline individual worms (Created by student researcher).

Curcumin Treatment decreases alpha synuclein aggregation in day 8 worms

ImageJ analysis of 60 images of day 8 worms revealed a decrease in integrated density in curcumin-treated groups. Day 8 worms that received curcumin treatment showed a mean integrated density of 10119691.60 ± 2932516.07 for 30 samples. Untreated Day 1 worms displayed a mean integrated density of 27991815.33 ± 11182272.96 for 30 samples. Therefore, treatment with curcumin decreased mean integrated density by about 36.2% for day 8 adult *Caenorhabditis elegans* ($p<.05$).

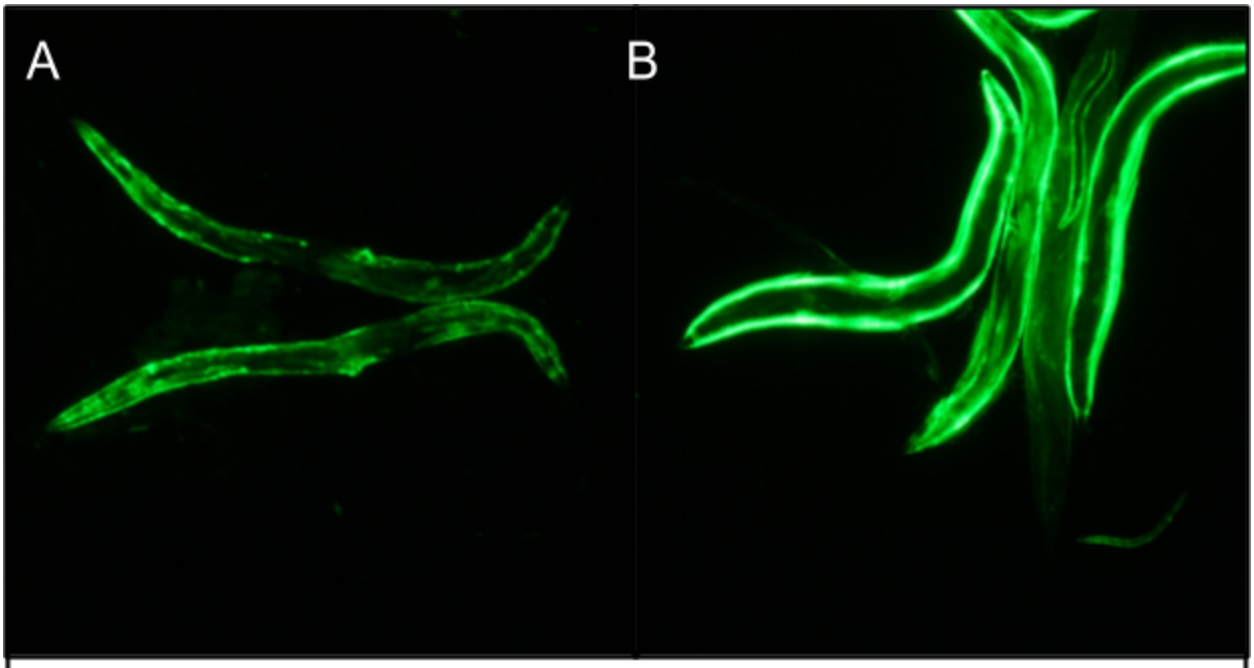

Fig. 7 Images of Adult Day 8 age synchronized NL5901 *C. elegans.* (A) Grown on NGM agar plates seeded with curcumin (concentration=20μM) and (B) worms grown on normal, unseeded NGM agar plates. ImageJ Integrated Density was used to measure level of aggregation via selection of individual worms using the freehand tool. Fluorescence intensity was measured as the Integrated Density for each of 30 worm samples, and average fluorescence values were taken. T-test analysis was conducted to determine that treated worms displayed significantly ($p<.05$) less alpha synuclein aggregation than their untreated counterparts (Created by student researcher).

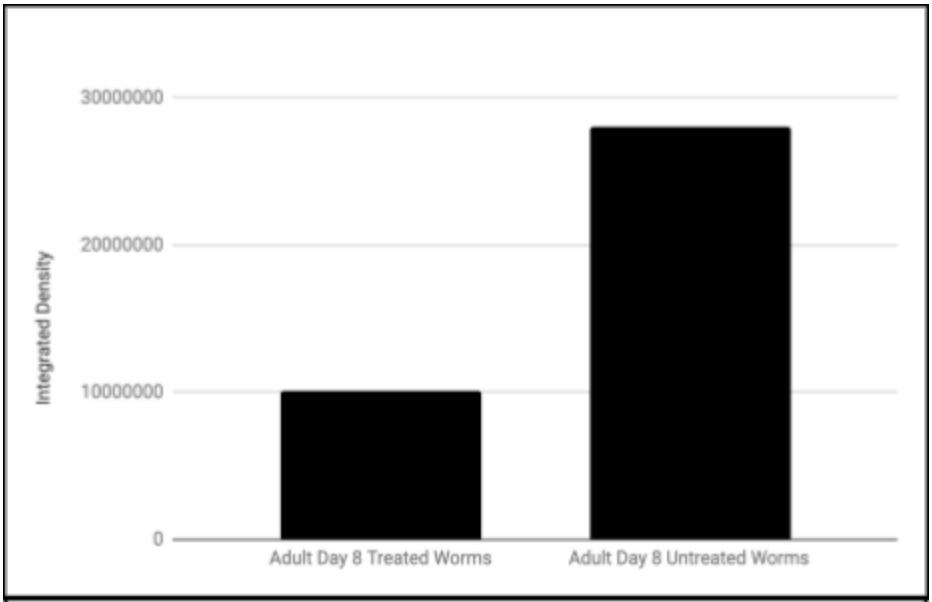

Fig. 8 Mean Integrated Density measurements for age synchronized Adult Day 8 NL5901 worms. Treated worms, grown on curcumin-seeded NGM agar plates (concentration = 20 μM), displayed a significantly lower mean Integrated Density value than untreated worms, grown on unseeded NGM agar plates. This lower level of mean Integrated Density suggests a lower level of fluorescence intensity in the Day 8 worms, which correlates with lower levels of alpha synuclein aggregation. Integrated Density measurements taken via ImageJ's Integrated Density measurement after using the freehand tool to outline individual worms (Created by student researcher).

Discussion

The expression of the fused alpha synuclein and YFP protein in body wall muscle cells was selected in the NL5901 mutant due to the large size of muscle cells, which allows for easier viewing of protein aggregation. Neuronal *Caenorhabditis elegans* cells are comparatively smaller than *C. elegans* muscle cells, which makes inclusion visualization via fluorescence microscope more difficult. Also, several other protein aggregation disease models have utilized *Caenorhabditis elegans* muscle cells, and found that neuronal effects are insignificant (van Ham *et. al* 2008).

The fluorescence observed in the fluorescence microscope is caused by the expression of YFP in the NL5901 *Caenorhabditis elegans* body cells (Bodicharla *et. al*, 2012). Since YFP is fused to alpha synuclein via the unc-54 promoter, at all locations of YFP there is also alpha synuclein presence. It is this fusion that allows the fluorescence intensity calculated via ImageJ to serve as an indicator of the presence and density of alpha synuclein aggregation in the muscle cells of *Caenorhabditis elegans*. Therefore, worms of higher fluorescence are known to have higher levels of alpha synuclein aggregation.

As previously mentioned, the fluorescence intensity of adult day 6 treated worms was significantly ($p<.05$) lower than the fluorescence intensity of adult day 6 untreated worms. Both groups of worms were maintained in identical conditions of 22ºC on NGM agar. Since the sole variable between the two groups was the seeding of experimental plates with curcumin, it is clear that the curcumin treatment is the cause of the decrease in fluorescence between the experimental and control groups. Therefore, since higher levels of fluorescence indicate a larger degree of alpha synuclein aggregation, it is reasonable to conclude that the phytochemical curcumin was able to decrease the levels of alpha synuclein aggregation in adult day 6 worms.

In adult day 8 worms, the calculated fluorescence intensity of the curcumin-treated *Caenorhabditis elegans* was significantly ($p<.05$) lower than the calculated fluorescence intensity of the untreated worms. Both groups were maintained in identical conditions, which means that the cause of difference in fluorescence intensity was curcumin treatment.

Since the untreated worms displayed higher levels of fluorescence than the treated worms, alpha synuclein aggregation was significantly ($p<.05$) decreased by the exposure of *C. elegans* to curcumin. Since curcumin treatment was able to ameliorate the aggregation of alpha synuclein in *Caenorhabditis elegans*, and alpha synuclein aggregation into Lewy bodies is a major cause of Parkinson's Disease in humans, it is reasonable to conclude that curcumin is a viable treatment option for Parkinson's Disease patients.

The exact biochemical reason for the decrease in alpha synuclein aggregation in *Caenorhabditis elegans* due to curcumin treatment is not yet known, but is hypothesized to be the upregulation of autophagic vessels during the hormetic response. The upregulation of autophagy would have increased the rate at which the nematodes were able to clear out toxic misfolded proteins in their body cells, which would then decrease the rate at which the alpha synuclein protein was able to build up in the *Caenorhabditis elegans* body muscle cells.

Further research would involve the corroboration of the results achieved in this study, through Western Blot analysis using antibodies targeting the YFP that is attached the the alpha synuclein in the aggregated worms (Maulik *et. al*, 2018). Verifying that the molecular pathway of curcumin-induced alpha synuclein aggregation prevention indeed utilizes an increase in autophagy is another method of confirming the presented lab results in future research. This confirmation can be achieved via utilization of *Caenorhabditis elegans* strain DCR4750, which exhibits GFP-tagged autophagosomes in worm neurons. Through the visualization of autophagosomes via Green Fluorescent Protein, the triggering of autophagy can be quantified using this strain as autophagosomes are signalled for production only during autophagy, and GFP tagging would allow visualization and quantification of autophagic vessels (Tsai *et. al*, 2010).

Future research could also involve the quantification of *Caenorhabditis elegans* health during curcumin treatment. An example of health measure includes the brood size and progeny of the worms, motility assays, and lipid staining treatments (Maulik *et. al*, 2018). The effect of curcumin on neuronal degeneration can also be measured, through using the strain UA196. This strain displays age-dependent neurodegeneration in the six anterior neurons of the worms, which have been shown to successfully model Parkinson's-related neurodegeneration in the past (Pujols *et. al*, 2018).

The full potential of curcumin to treat the symptoms of Parkinson's disease has not yet been realized, however in showing that it can decrease the levels of alpha synuclein aggregation in *Caenorhabditis elegans*, a new avenue of neurodegenerative research has been opened for exploration.

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