Effect of natural compounds curcumin and nicotinamide on α-synuclein accumulation in a C. elegans model of Parkinson’s disease

Rohan Mehrotra1*, Isha Mehrotra1*, Jason Lee1
1Lynbrook High School, San Jose, CA
*Authors contributed equally to this work

Summary
Parkinson’s disease is a neurodegenerative disease that causes the death of dopamine-producing neurons. It is associated with the accumulation of a protein called α-synuclein, which is responsible for the death of the neurons and causes severe motor disorders. Current drugs have significant side effects and only treat symptoms rather than the actual disease. Our study aims to explore the anti-Parkinsonian effects of curcumin and nicotinamide, which are two compounds derived from natural sources. Curcumin and nicotinamide were chosen for their health benefits and properties that suggest anti-Parkinsonian potential. In our study, we used the model organism C. elegans, a nematode. Our strain expresses human α-synuclein fused to yellow fluorescent protein. The study examined the effect of curcumin and nicotinamide on the fluorescence intensity of α-synuclein in C. elegans and compared it to the effect of Levodopa (the commercial drug most commonly prescribed to Parkinson’s patients). We used two methods to measure fluorescence. In our first method, the worms were imaged after treatment under a fluorescence microscope, and fluorescence was quantified using ImageJ. In the second method, the fluorescence of the worms was measured after treatment using a microplate reader. Our study showed that curcumin and nicotinamide reduce the fluorescence intensity of α-synuclein as effectively as levodopa in C. elegans. This suggests that curcumin and nicotinamide may affect α-synuclein levels in other organisms and should be further investigated as treatments for Parkinson’s disease. These findings also encourage further investigations on other natural compounds as possible therapies against Parkinson’s disease.

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Introduction
Parkinson’s disease (PD) is a progressive, neurodegenerative disorder that affects over 10 million people worldwide and mainly affects people over the age of 60. PD is a type of motor system disorder, which means that it involves the loss of neurons that produce dopamine, a neurotransmitter associated with motor activities (1). The major region of the brain affected by the disease is the substantia nigra, which controls balance and movement. Thus, the apparent symptoms of PD are primarily motor or movement disorders. The four main symptoms of Parkinson’s disease are tremor (trembling), rigidity (stiffness), bradykinesia (slowness of movement), and postural instability (impaired balance and coordination) (1). Advanced stages of Parkinson’s also involve cognitive problems like dementia, depression, and anxiety (2).

The hallmark of PD in the neurons of almost all patients, including patients with both familial and sporadic PD, is the development of Lewy bodies (3). Lewy bodies are toxic intracellular aggregations of α-synuclein, a protein that is encoded by the SNCA gene and is abundant in the human brain. The function of α-synuclein in the healthy brain is not well understood. In PD, however, α-synuclein misfolds, accumulates, and forms Lewy bodies. These toxic, accumulated aggregates interfere with neuronal function and are believed to contribute to and induce the degeneration of dopaminergic (DA) neurons (3). The underlying cause of DA neuronal death in PD is still unclear, as several factors, including mitochondrial dysfunction and oxidative stress, are also believed to play key roles in neurodegeneration (4,5). However, there is strong evidence that α-syn-mediated neuronal degeneration is the primary factor responsible for the pathogenesis of PD (4,5).

A recent landmark study by Nemani et al. found that dopamine neurons that overexpress α-synuclein show less synaptic vesicle exocytosis, providing a plausible hypothesis for the mechanism of syn-mediated neuronal degeneration (6). Specifically, α-synuclein blocks vesicles that store dopamine from releasing their neurotransmitters. This inhibits synaptic transmission and prevents neurons from firing. Over time, this impaired functioning would cause the degeneration and death of dopamine neurons (6). Thus, treatments that reduce the accumulation of α-synuclein should be explored and hold great promise for the treatment of Parkinson’s disease.

Currently, there is no effective cure for Parkinson’s disease. Drugs that are used today like levodopa, dopamine agonists, and monoamine oxidase B only delay or relieve the motor symptoms of PD, rather than treat the underlying cellular causes of neurodegeneration (7). Furthermore, many drugs
used today have significant side effects. For example, levodopa, the most widely used treatment for over 30 years, has side effects including abnormal thinking, agitation, anxiety, confusion, dizziness, hallucinations, weakness, and numbness (8). The group of drugs called dopamine agonists cause drowsiness, hallucinations, insomnia, nausea, and constipation (9). Thus, there is a great need for treatments that address the underlying cause of Parkinson's without major side effects. Natural compounds that are consumed in high concentrations from food are less likely to produce harmful side effects. Due to this property of natural compounds, recent research has focused on finding natural compounds that can treat the cellular perturbations that underpin diseases with fewer side effects (10).

Several natural compounds are being investigated for their neuroprotective effects and could be viable treatments for neurodegenerative diseases (11). However, many natural compounds are still unexplored. In this study, we analyzed the therapeutic effects of nicotinamide and curcumin, two natural compounds, in a Caenorhabditis elegans model of PD. Nicotinamide is produced from vitamin B3 in the human body (12). It is found in niacin-rich foods like lean meats, fish, nuts, and legumes like beans or lentils. Nicotinamide has previously shown promise in the prevention of various diseases including type 1 diabetes, esophageal cancer, throat cancer, and mouth cancer (12). Curcumin is a substance found in turmeric, a spice (13). It is commonly used in India and Asia for treating health conditions. Preliminary research has shown that curcumin may possess several health benefits, including protection against some skin diseases, stomach ulcers, high cholesterol, and treatment of upset stomach, diabetes, HIV, and other viral infections (13). In addition to the aforementioned potential health benefits of nicotinamide and curcumin, both compounds have been shown to have neuroprotective properties (14,15). Due to these known therapeutic qualities of nicotinamide and curcumin, we hypothesized that these compounds have considerable potential for the treatment of PD.

We employed a transgenic C. elegans strain as a model to evaluate the effect of nicotinamide, curcumin (natural compounds), and levodopa (current drug) on Parkinson's disease (16). C. elegans offers several advantages as a model to study the effect of drugs on Parkinson's disease (17). First, it is small, has a short life cycle, and is inexpensive to grow. Second, it has only eight dopaminergic neurons, which have been extensively mapped. Third, C. elegans has homologs of several critical genes associated with Parkinson's disease (17).

Specifically, we utilized a transgenic strain (NL5901) expressing human α-synuclein fused to yellow fluorescent protein (YFP) as a model to study the effect of potential therapeutic compounds. Because C. elegans are transparent, it is easy to monitor the aggregation of fluorescently-tagged α-synuclein in the worms and precisely measure the levels of α-synuclein accumulation. The NL5901 strain represents an effective way to model PD. Because research suggests that increased α-synuclein accumulation is responsible for DA neuronal degeneration, monitoring the levels of α-synuclein in the C. elegans model is a valid surrogate for measuring the efficacy of PD treatments.

It is important to note that α-synuclein does not induce neurodegeneration in this model because it is expressed in the muscle cells, so the worms do not exhibit PD-like symptoms. However, this model is valuable as it is a tractable system to assess the effects of the compounds
on α-synuclein aggregation in vivo.

The goal of our project was to determine whether nicotinamide and curcumin could reduce the levels of α-synuclein in the transgenic worm model of PD. We assessed the effects of nicotinamide and curcumin on α-synuclein accumulation in the C. elegans worms and compared them to the effects of levodopa. Our hypothesis was that nicotinamide and curcumin would reduce the levels of α-synuclein due to the known properties and purported health benefits of these two natural compounds.

Results

In both Trial 1 and Trial 2, NL5901 C. elegans groups were treated with nicotinamide, curcumin, levodopa, or DMSO control for 96 hours.

In Trial 1, α-synuclein protein accumulation in the worms was assayed after treatment by fluorescence microscopy and quantified using ImageJ (Table 1). Treatment with all three compounds robustly and significantly reduced fluorescence intensity of α-synuclein (tagged with YFP) compared to the control (Figure 1, Figure 2). The most effective compound was nicotinamide, followed by curcumin, followed by levodopa (Table 1). The YFP-tagged α-synuclein was uniformly expressed in the body walls of the worms. As expected for NL5901 worms (16), no clear aggregates formed in the worms, and α-synuclein accumulated in a diffuse manner in the body wall of the worm (Figure 1). The worms grew in size from the L3 (larval) stage to the adult stage over the duration of the experiment (96 hours). Excluding normal growth, there were no distinct locomotive or morphological changes observed in the animals over the course of treatment, as expected.

Additionally, in Trial 2, we directly measured fluorescence in groups of worms before and after 96 hours of treatment using a plate reader. The results from Trial 2 were more precise than Trial 1, likely because quantification of α-synuclein through ImageJ can introduce error due to the morphological and biochemical variation between individual worms. Treatment with all three compounds again showed a robust and significant reduction in the fluorescence intensity of α-synuclein compared to the DMSO control (Figure 3). By this method, the most effective compound was levodopa, followed by curcumin, followed by nicotinamide (Table 2).

Table 1. Effect of compounds on YFP fluorescence intensity assayed by fluorescence microscopy. The percent decrease in mean fluorescence intensity of DMSO control, nicotinamide, curcumin, and levodopa-treated NL5901 groups after 96 hours of treatment is shown.

<table>
<thead>
<tr>
<th></th>
<th>DMSO Control</th>
<th>Nicotinamide + DMSO</th>
<th>Curcumin + DMSO</th>
<th>Levodopa + DMSO</th>
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<tbody>
<tr>
<td>96 hours</td>
<td>2%</td>
<td>64.7%</td>
<td>56.1%</td>
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Discussion

The results of our experiments confirmed our hypothesis, as nicotinamide and curcumin effectively decreased the accumulation of α-synuclein in the NL5901 worms, which are a useful PD model. The two natural compounds were also comparable with levodopa in reducing α-synuclein in the worms.

The final concentration of each compound in the worms’ agar media was 0.704 mM. This is equivalent to a concentration of 85.97 mg/L nicotinamide and 267.87 mg/L curcumin. These concentrations are comparable to the doses of each compound that one can receive from consuming daily foods. For example, a 3-oz serving of tuna fish, which is high in nicotinamide, contains a niacin concentration of about 211.5 mg/L (18) (niacin amounts can be used to approximate nicotinamide amounts because niacin is converted into nicotinamide in the body (19)). In addition, turmeric powder, the South Asian spice that is high in curcumin, contains a curcumin concentration of 20331 mg/L (20,21). Although the concentration of curcumin is extremely high, turmeric is a powder, so it is generally added to dishes in small quantities during daily practice. For example, a sample recipe for turmeric-infused tea calls for the addition of ½ teaspoon of turmeric (equivalent to 0.01 cups) to 1 cup of tea, which would bring the final concentration of curcumin in the tea to 201 mg/L (22). Although further studies are necessary to confirm that normal foods can provide enough of each compound for therapeutic effects, the concentrations used in our study are comparable to concentrations in foods in daily life.
Strong evidence suggests that accumulation of α-synuclein is responsible for the degeneration of dopamine-producing neurons (3,4,5,6). Thus, reducing α-synuclein aggregation in DA neurons, which is thought to contribute to the death of those neurons, is a promising strategy for PD therapy. Our results therefore demonstrate that these natural compounds are good candidates for further study as viable treatments for other PD animal models and, ultimately, for PD-afflicted patients.

There are two major implications of these results. First, it may prove to be beneficial for Parkinson's patients to consume foods that are high in nicotinamide and curcumin. The potential of this application is strengthened by the fact that the compound concentrations we used in the experiments were comparable to those found in common foods. However, further studies are necessary to determine if these effects are conserved in higher-order PD models and if foods high in these compounds have benefits for human patients in daily practice.

Second, nicotinamide and curcumin should be studied further to determine whether they can be used as therapeutics in their purified forms. Further experimentation should be conducted to determine whether these compounds can reduce α-synuclein levels in higher-order PD models and if they are safe and remain effective over prolonged, high-level consumption.

This study indicates that the two tested natural compounds are similar in effectiveness to the commercial PD drug, levodopa, in reducing α-synuclein aggregation as quantified by fluorescence in a transgenic nematode model of PD. This study additionally encourages further investigations on natural compounds and foods high in them, as possible avenues for the treatment of Parkinson’s disease.

It should be noted that the comparative effects of levodopa, curcumin, and nicotinamide on α-synuclein accumulation were different in Trial 1 and 2. In Trial 1, nicotinamide was most effective, followed by curcumin and levodopa. In Trial 2, levodopa was most effective, followed by curcumin and nicotinamide. The differences in these results likely arose from experimental variation. We note that the variation in the Trial 1 data was insufficiently small to conclude that the differences in the effectiveness of each compound were statistically significant. Thus, we conclude that curcumin and nicotinamide were able to reduce α-synuclein aggregation in the C. elegans model, but we cannot make firm conclusions as to which compound was more effective.

There are several properties of both nicotinamide and curcumin that may explain why they are effective in decreasing α-synuclein. In a literature review, Mythri et al. wrote that curcumin has been shown to prevent mitochondrial dysfunction (14). Research has shown that mitochondrial dysfunction can directly lead to α-synuclein aggregation (23). Curcumin may also have reduced α-synuclein accumulation via inhibition of mitochondrial dysfunction. Curcumin has also been shown to target protein aggregations, allowing it to directly reduce α-synuclein (14). Furthermore, curcumin has strong clinical potential, as it can cross the blood-brain barrier, and preliminary data suggests that it is not toxic in humans even at high doses (14).

Past research has demonstrated that nicotinamide prevents oxidative stress in cells (15) Research has shown that oxidative stress, like mitochondrial dysfunction, can lead to aggregation of α-synuclein (24) Nicotinamide may have decreased α-synuclein aggregation by preventing oxidative stress. Moreover, research has also shown that nicotinamide prevents mitochondrial dysfunction (25). Mitochondrial dysfunction increases α-synuclein accumulation, so counteracting this is a second possible mechanism by which nicotinamide reduces α-synuclein.

A possible limiting factor in this study was the strain used for the transgenic model, NL5901. NL5901 worms express YFP-tagged α-synuclein under control of the unc-54 promoter, which causes expression in the body wall muscle cells (15). The model is useful to measure α-synuclein levels, but cannot be used to directly investigate DA neuron health. This is a limitation.

<table>
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<tr>
<th>Table 2. Effect of compounds on YFP fluorescence intensity assayed by microplate reader. The percent decrease in mean fluorescence intensity of DMSO control, nicotinamide, curcumin, and levodopa-treated NL5901 groups after 96 hours of treatment is shown.</th>
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<tr>
<td>Treatment</td>
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<td>96 hours</td>
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| Figure 3. Effect of compounds on YFP fluorescence intensity assayed by microplate reader. The mean YFP intensity of α-synuclein in NL5901 C. elegans after 96 hours of treatment with nicotinamide or curcumin is shown. Fluorescence intensity was quantified with a plate reader. The worms were plated onto each treatment condition in triplicate, with five worms in each well. The data represent the mean ± SD (n = 3). Statistical significance is shown with asterisks (* p < 0.05 ). |
because Parkinson's disease in humans is a disease of DA neurons. Although this study did show that the compounds could biochemically reduce α-synuclein in vivo, further studies should be done to demonstrate that the compounds can also prevent α-synuclein-mediated DA neuronal death in other PD models.

**Methods**

*C. elegans Strain*

The transgenic *C. elegans* strain NL5901 [unc-54p::alphasynuclein::YFP + unc-119(+)] was used in this experiment. NL5901 expresses human α-synuclein fused to Yellow Fluorescent Protein (YFP) in the body wall muscle cells. The protein is expressed under the control of the unc-54 promoter, which drives protein expression in the body wall muscle cells. NL5901 was engineered by Tjakko van Ham of the Hubrecht Laboratory in the Netherlands. The strain was kindly provided by the CGC (Caenorhabditis Genetics Center). Standard protocols were followed for general worm maintenance (26, 27, 28, 29).

**Preparation of food**

OP50 *E. coli* was prepared as a food source for the worms. A starter plate for OP50 *E. coli* was obtained. One colony from the OP50 *E. coli* starter plate was inoculated in 200 mL of LB broth. The bacteria was incubated and grown overnight at 37°C for growth.

**Preparation of compounds: curcumin, nicotinamide, levodopa**

The compounds assessed in the experiment were curcumin and nicotinamide (the natural compounds) in addition to levodopa (the commercial Parkinson's drug). The mass of each of the three compounds needed to create a 100 mM concentration in 10 mL of DMSO, equivalent to 0.001 moles of each compound, was calculated (DMSO is a common biological solvent). Each compound wasweighed to the appropriate amount on a precision scale, then dissolved in 10 mL of DMSO. A stock solution of each compound was therefore produced, with each compound dissolved in 10 mL of DMSO to a 100 mM concentration.

**Trial 1 Methods**

*Preparation of plates*

A bottle of NGM (Nematode Growth Media) agar was microwaved until boiling, then cooled to 60°C. 7 mL of the molten NGM agar was added to each of three tubes. 49.3 uL of the DMSO/compound stock solution, equivalent to 0.0007 moles of the solution, was diluted with the 7 mL of NGM in each tube to the concentration of 100 mM. The final concentration of each drug in NGM agar was 0.704 mM. The NGM mixed with the DMSO/compound solution was poured into petri plates to make the supporting bed for worms. Plates for each compound were produced with each plate containing a 100 mM concentration of the compound stock solution in NGM agar. One mL of OP50 bacteria grown in LB broth was then centrifuged at 15000 rpm for 3 minutes to create a bacterial pellet. OP50 bacteria was pipetted from the pellet onto the NGM plates, then spread to form a bacterial lawn.

**Treatment of worms with curcumin, nicotinamide, levodopa**

A starter plate of NL5901 worms was obtained from the CGC. A piece of agar was chunked onto each of the three experimental plates. This allowed the worms to move onto the plates and gain exposure to the compounds. The worms were allowed to grow on the compound-treated plates for 96 hours of data collection.

**Quantitative assay of α-synuclein accumulation**

Accumulation of α-synuclein protein was assayed in control NL5901, nicotinamide-treated NL5901, curcumin-treated NL5901, and levodopa-treated NL5901 worms. For imaging, worms from each plate were transferred to glass slides and sealed with a coverslip for immobilization. Worms were then imaged on an AxioVision fluorescence microscope using an iPhone 6 camera. Imaging was performed at 0 hours and 96 hours after treatment to monitor the accumulation of α-synuclein. Worms were imaged at advancing life stages as the experiment progressed from L3 larvae at 0 hours to adult at 96 hours, so that the worms imaged had been exposed to the compound for the entire duration of the experiment.

**Data analysis**

The accumulation of α-synuclein for all data points was quantified using ImageJ Software. For each worm, the mean fluorescence of the worm and mean fluorescence of the background was calculated. The final fluorescence value was calculated by the formula: Mean worm fluorescence - Mean background fluorescence. To calculate mean fluorescence, the average fluorescence of three worms was calculated. This was done for each group of worms at the two time points. Thus, the fluorescence of 48 total pictures (12 pictures for each group of worms) was quantified to generate 16 data points (four data points per group of worms).

**Statistics**

Statistical significance between the DMSO control and each compound group was analyzed with an unpaired, two-tailed, two-sample, unequal variance t-test (p<0.05). Statistical significance between fluorescence at 0 and 96 hours was also analyzed with an unpaired, two-tailed, two-sample, unequal variance t-test (p<0.05). Graphs were produced and significance analyzed using GraphPad Prism software.

**Trial 2 Methods**

The same methods were used as in Trial 1 to add the compounds and *E. coli* food to the NGM agar. However, in Trial 2, the NGM agar was poured into a microplate instead of a petri plate. In each well, 300 uL of NGM was added. Four of the wells were used for control,
nicotinamide, curcumin, and levodopa worms. Each well contained exactly five worms. At 0 hours and 96 hours, the fluorescence was directly measured using a BioTek Synergy plate reader machine.

Rationale for Utilizing Two Methods
Different methods were used to increase the accuracy of the results. In Trial 1, the amount of a-synuclein was measured in different worms throughout the experiment, because worms were randomly selected for measurement during each data collection. However, in Trial 2, the amount of a-synuclein was measured in the same five worms throughout the experiment, as the same worms were measured for their protein aggregation during all data collections.

Statistics
Significance was analyzed with an unpaired, two-tailed, two-sample, unequal variance t-test (p<0.05). Statistical significance between fluorescence at 0 and 96 hours was analyzed with a paired, two-tailed, two-sample, unequal variance t-test (p<0.05). (A paired t-test was used because fluorescence was measured in the same group of worms at 0 and 96 hours). Graphs were produced and significance analyzed using GraphPad Prism software.

References


