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REPLICATION

NOVEL-RESULT

Pyrroloquinoline quinone influences intracellular alpha-synuclein aggregates

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Abstract

Parkinson's disease (PD) is an irreversible neurodegenerative disorder clinically manifesting in uncontrolled motor symptoms. There are two primary hallmark features of Parkinson's disease—an irreversible loss of dopaminergic neurons of the substantia nigra pars compacta and formation of intracellular insoluble aggregates called Lewy bodies mostly composed of alpha-synuclein. Using a clinical improvements-first approach, we identified several clinical trials involving consumption of a specific diet or nutritional supplementation that improved motor and nonmotor functions. Here, we aimed to investigate if and how pyrroloquinoline quinone (PQQ) compound disrupts preformed alpha-synuclein deposits using SH-SY5Y cells, widely used Parkinson's disease cellular model. SH-SY5Y neuroblastoma cells, incubated in presence of potassium chloride (KCl) to induce alpha-synuclein protein aggregation, were treated with PQQ for up to 48 hr. Resulting aggregates were examined and quantified using confocal microscopy. Overall, nutritional compound PQQ reduced the average number and overall size of intracellular cytoplasmic alpha-synuclein aggregates in a PD cellular model.

Keywords: Alpha-synuclein; α-synuclein; Lewy body; nutrition; Parkinson's disease; pyrroloquinoline quinone

Introduction

Parkinson's disease is a physically restricting, incurable, and progressive neurodegenerative disease. Patients with Parkinson's disease suffer from increasing and irreversible loss of dopamine due to dopaminergic neuron cell death leading to loss of motor function, manifesting as resting tremors, muscle rigidity, and bradykinesia (Alberio et al., 2012). As Parkinson's disease worsens, everyday activities become progressively compromised and increasing care level is needed.

The aggregation of alpha-synuclein has been identified in both familial and sporadic forms of Parkinson's disease and is the main protein found in Lewy body (LB) pathology (Fields et al., 2019). Molecular mechanisms of alpha-synuclein toxicity in the cells include an increase in oxidative stress (Fields et al., 2019) and damage to the cell membrane structure through the formation of fibrils and pore-like structures (Chaudhary et al., 2014), leading to disturbance in cellular homeostasis and increase in cellular stress. Recent reviews have highlighted that specific cellular pathways may trigger changes in cellular homeostasis leading to dopaminergic cell death, including cellular oxidative damage, mitochondrial dysfunction, inflammation, the breakdown of cellular mechanisms such as an impairment of

protein degradation via chaperone-mediated autophagy and the aggregation of the alpha-synuclein protein (Gonzalez-Hunt & Sanders, 2021; Kalia & Lang, 2015; Pang et al., 2019). Overall, there appears to be an interplay of alpha-synuclein misfolding and/or aggregation and the cellular pathways leading to the death of dopaminergic neurons. Therefore, identifying a compound that targets and/or fragments the aggregation may be key in future treatment strategies for Parkinson's disease.

The gold standard treatment for Parkinson's disease is pharmacological and based on dopamine replacement therapy using a dopamine precursor agent such as L-Dopa. Unlike dopamine, L-Dopa can cross the blood–brain barrier and metabolize to dopamine in the central nervous system and the peripheral tissues. L-Dopa is often administrated with peripheral decarboxylase inhibitors such as carbidopa to prevent L-Dopa's peripheral conversion to dopamine, allowing more L-Dopa to cross over the blood–brain barrier for dopamine conversion (Meiser et al., 2013). However, pharmacological treatments carry significant side effects, risk, and diminished response rates as the disease progresses (Singh et al., 2007) leaving limited treatment options available for Parkinson's disease patients.

Currently, there are limited targeted dietary and/or nutritional interventions integrated into clinical practice to manage Parkinson's disease symptoms in patients. Dietary interventions focus on managing the symptoms of comorbidities such as constipation, weight loss, and malnourishment (Barichella et al., 2009). However, several clinical trials and in vitro studies identified specific diets and nutrient compounds that may assist in improving both motor and nonmotor functions. For instance, ketogenic diet trials have shown an improvement in both motor and nonmotor symptoms (Phillips et al., 2018; Vanitallie et al., 2005), supplementation with potent intracellular antioxidants CoQ10 and glutathione, often depleted in Parkinson's disease patients, showed minor improvement in motor symptoms (Chang & Chen, 2020; Mischley, 2011; Mischley et al., 2012) and food containing certain nutrient compounds such as carotenoids, vitamin E, sulforaphane, Omega-3, resveratrol, epigallocatechin-3-gallate, and caffeine might offer important neuroprotective properties, including the potential to alter the underlying pathological cellular and molecular changes identified in Parkinson's disease (Seidl et al., 2014). Nutrients including selenium, vitamins A, C, and E may serve as potent antioxidants protecting the dopaminergic cell from oxidative damage to preserve dopamine-producing neurons (Singh et al., 2007).

Interestingly, in a dietary study where participants were given broad beans to consume as part of their daily diet, improvements in motor function and increased levels of endogenous levodopa were observed similar to levels achieved with L-Dopa therapy (Rabey et al., 1993). On further investigation, it was also found that broad beans contain high levels of a nutritional compound pyrroloquinoline quinone (PQQ) (Kumazawa et al., 1995), which has shown to modify alpha-synuclein aggregation in vitro (Kobayashi et al., 2006).

PQQ is a water-soluble organic molecule found in varying quantities in vegetables, fruits, and beverages such as tea. PQQ is a vitamin-like compound with combined chemical properties similar to ascorbic acid, vitamin B_2, and vitamin B_6, but with a nutritional requirement similar to folate and biotin (Nakano et al., 2012). In animal studies, PQQ has been demonstrated to be highly absorbable, predominantly in the lower intestine, and nontoxic in both animals and humans, indicating low or no side effects (Rucker & Chowanadisai, 2009). Recent studies have demonstrated that PQQ permeability to the blood–brain barrier is enhanced using esterification processes (Tsukakoshi et al., 2018). Importantly for molecular events leading to Parkinson's disease, PQQ was shown to have an anti-aggregation effect on the alpha-synuclein protein in vitro (Kobayashi et al., 2006) and inhibits fibril formation in both a full length and C-terminal deleted mutant alpha-synuclein in vitro (Yoshida et al., 2013). However, none of the other alpha-synuclein studies had been conducted in the PD-relevant cellular context.

Our study demonstrated that the addition of PQQ to preformed alpha-synuclein aggregates can reduce the number and size of these aggregates in a Parkinson's disease SH-SY5Y cellular model. This study is the first to show PQQ aggregation-modifying properties in a Parkinson's disease-specific cellular model. It confirms previous in vitro and clinical trial reports establishing this nutritional compound and food containing PQQ as an important player in the regulation of Parkinson's disease symptoms and possible novel treatment approach.

Methods
Materials
SH-SY5Y neuroblastoma cells (94030304-1VL; Sigma). Hams F-12 nutrient mixture (N48880), Eagles Minimum Essential Medium (EMEM; M2279), glutamine (GT513), nonessential amino acids (M7145), pyrroloquinoline quinone (D7783-1MG) were purchased from Sigma-Aldrich. Monoclonal antibody to alpha-synuclein (ab27766) was purchased from Abcam, secondary goat anti-mouse Alexa Fluor 488 IgG antibody (A-11001) was from ThermoFisher Scientific and Foetal Bovine Serum (FBS, SFBSF8) from Bovogen. Pyrroloquinoline quinone (PQQ) was dissolved in 1 M sodium hydroxide (NaOH) solution to prepare a stock concentration of 50 mM, and 50 μM PQQ was used as the final concentration.

Methods
Cell culture
The SH-SY5Y neuroblastoma cells were maintained in the following cell growth media mix—EMEM, Ham's F12 nutrient mixture, 1% glutamine, 1% nonessential amino acids, and 15% FBS. Cells were maintained in a humidified incubator at 37 °C supplied with 5% CO_2.

Experimental procedures
Cell seeding
24 hours (hr) prior to the experiment, cells were cultured on glass coverslips at 20,000 cells per well of a 12-well multiwell dish. Each experiment contained the following groups in triplicates: no treatment, 50 mM KCl treatment, 50 μM PQQ treatment, 50 mM KCl followed by 50 μM PQQ treatment.

PQQ and KCl treatment
Cells were treated with 50 μM PQQ for 1 h, followed by treatment with 50 mM KCl to the appropriate samples for 60 minutes (min). After 60 min, the PQQ/KCl containing media was decanted and replaced with growth media containing PQQ as described and incubated further for 24 or 48 hr.

Immunofluorescence assay
At the 24- and 48-hr time points, cells were fixed using 4% paraformaldehyde (prepared in 1 X PBS; PFA/PBS) and permeabilized with 0.1% Triton X-100. Fixed coverslips were stained with primary mouse anti-alpha-synuclein antibody (optimized at 1:50 dilution) and secondary goat anti-mouse Alexa 488 IgG antibody. Hoechst DNA stain diluted in 1X PBS was used for nuclear staining. The coverslips were mounted onto microscopy slides using Fluorescence mounting medium (Dako) and analyzed using confocal microscopy.

Confocal microscopy
Digitized fluorescent cellular images were collected using a Nikon Eclipse Ti confocal laser-scanning microscope, with a Nikon 60x/1.40 oil immersion objective. Images were captured at an optical thickness of 0.68 μm and an optical resolution of 0.12 μm. An average of 10–15 images for each treatment group from two independent experiments were captured, resulting in approximately 250 images.

Image analysis
Image analysis was performed in ImageJ v1.51—images were opened as a hyperstack, split channel color composite, 16-bit image. Each color composite image was separated into two multilevel channels (alpha-synuclein and nucleus), and both channels were then merged into one composite image while preserving the original multilevel composite channels. To determine the aggregates' approximate size, a scale was set in ImageJ at 1 pixel to 0.21 μm, and an outline of the aggregates was traced. The ImageJ preset function

was used to calculate the approximate perimeter size of the aggregate. Aggregate size data and the total number of aggregates were captured for both the 24 and 48-hr per treatment group and entered into Prism GraphPad v9 to produce a scatter plot and determine differences between groups.

Images were discounted if there was either insufficient immunofluorescence staining of the alpha-synuclein or the nucleus, if cells had not shown sufficient growth and proliferation, or if cells were out of focus. Cells were discounted from analyzes if the DNA staining did not define a distinct nucleus or if there were morphological abnormalities, such as condensing or blebbing of the cell nucleus.

Results

Confocal microscopy revealed two general patterns of alpha-synuclein localisation at both time points investigated—generalized diffusion surrounding the cell nucleus and aggregates predominantly located near the nucleus of the cell (arrows, Figure 1a,b).

To objectively determine the number of alpha-synuclein aggregates per cell, images were analyzed using ImageJ by counting the total number of aggregates and normalizing this count to the total number of cells in the image for each sample or by measuring the size of the individual aggregate (Figure 2a,b). The highest percentage of aggregates was observed in 50 mM KCl only treated samples at both time points, 25.71% and 6.48% compared to 1.47% and 0.66% in no treatment samples at 24 h and 48 h, respectively. A three-fold decrease in the number of aggregates was observed in cells treated with PQQ only at both time points, 8.43% and 1.65% at 24 h and 48 h, respectively. This decrease was two-fold in cells treated with PQQ and stimulated with 50 mM KCl compared to control, 11.40% and 3.49% at 24 h and 48 h, respectively (Figure 2).

The same images were used to determine the average size of all aggregates (Figure 2). A measurable difference in mean aggregate size between the 24 and 48 h control and experimental samples was observed. Across all samples, 24 h samples contained on average smaller aggregates (Figure 2a). The mean aggregate size in the presence of PQQ only or PQQ + KCl was 1.84 μm and 2.51 μm, respectively, compared to 3.05 μm in the presence of KCl only at 24 h. The same trend was evident at 48 h with a decrease in mean aggregate size in the presence of PQQ (1.94 μm; PQQ only) and 2.85 μm (PQQ + KCl), compared to KCl only (3.88 μm). In comparison to their controls, in samples incubated with 50 mM KCl, the mean aggregate size in the presence of PQQ + KCl decreased to 17.7% and 26.5% at 24 h and 48 h, respectively (Figure 2a,b).

Overall, the cells treated with PQQ without or with KCl consistently demonstrated an overall reduction in the number and mean aggregate size of alpha-synuclein at both 24- and 48-hr compared to cells that were not treated with PQQ.

Discussion

In the current study, we used an established Parkinson's disease cellular model, neuroblastoma SH-SY5Y cell line, to demonstrate the reduction in number and size of preformed intracellular alpha-synuclein aggregates at 24 and 48 h post-treatment with PQQ.

PQQ was first identified in 1979 as a redox cofactor for bacterial dehydrogenase. Although PQQ is not synthesized in humans, it is found in trace amounts in tissues due to absorption from everyday food, such as broad beans, green peppers, spinach, fermented soybeans, oolong tea, green tea, papaya and kiwi fruit (Kumazawa et al., 1995). The nutritional requirement for PQQ from a dietary perspective is not defined, but animal studies that modified diets to omit PQQ resulted in impaired growth, immune dysfunction, and fertility issues (Murray, 2018).

In humans, the nutritional importance of PQQ and the possible clinical applications are increasingly emerging with a primary focus on PQQ supplementation is its potential to have a pharmacological-like role in human disease. In human and animal studies, PQQ is observed to have positive physiological benefits for various diseases including dementia, neurodegenerative disorders, Parkinson's disease, cardiovascular disease, chronic inflammation, insulin resistance, mood disorders, and accelerated aging
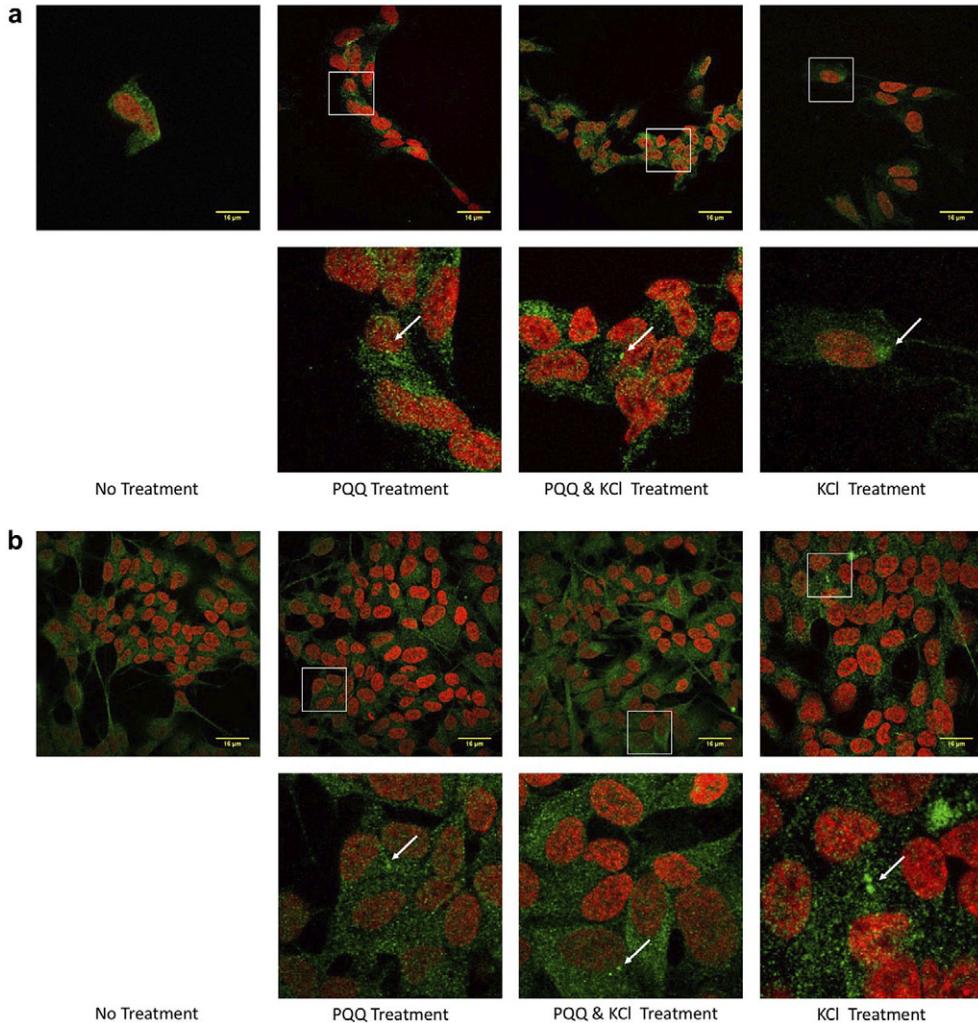
Figure 1. Images of alpha-synuclein aggregates show morphology change in the presence of PQQ. SH-SY5Y cells were incubated in the presence or absence of 50 mM PQQ for 1 hr and then for a further hour with 50 mM KCl treatment as appropriate for the sample to induce alpha-synuclein aggregation. After KCl treatment, the cells were washed into full cell media containing 50 mM PQQ ("PQQ treatment "and "PQQ & KCl treatment") or full cell media only ("no treatment" and "KCl treatment") for 24 hr (a) or 48 hr (b). The cells were fixed and then stained using anti-alpha-synuclein antibody and secondary goat anti-mouse Alexa Fluor 488 IgG antibody. Hoechst 3342 diluted in 1X PBS was used for nuclear staining (shown in red). The images were taken with a Nikon Ti Eclipse confocal laser-scanning microscope and NIS Elements AR software. Scale bar: 16 μm.

(Akagawa et al., 2016). Animal research models have identified the benefits of PQQ treatments to include overall improvement and even reversal of cognitive impairment, stimulation of nerve growth factors for cell proliferation, protecting cells from oxidative stress and neurotoxicity caused by cerebral ischemia, reduction in neurotoxin-induced injury to the brain (Zhang et al., 2006, 2009, 2011) and protecting nerve cells from beta-amyloid damage in Alzheimer's disease (Zhang et al., 2009). Molecular mechanisms underpinning these observed phenotypes include an increase in mitochondrial energy production, promotion of mitochondrial biogenesis, and AMP kinase activity which serve as a master switch for energy production (Cheng et al., 2020).

Human clinical studies using PQQ as a supplement showed an increased presence of the compound in blood and reduced levels of inflammatory markers C-reactive protein (CRP) and interleukin 6 (IL-6)
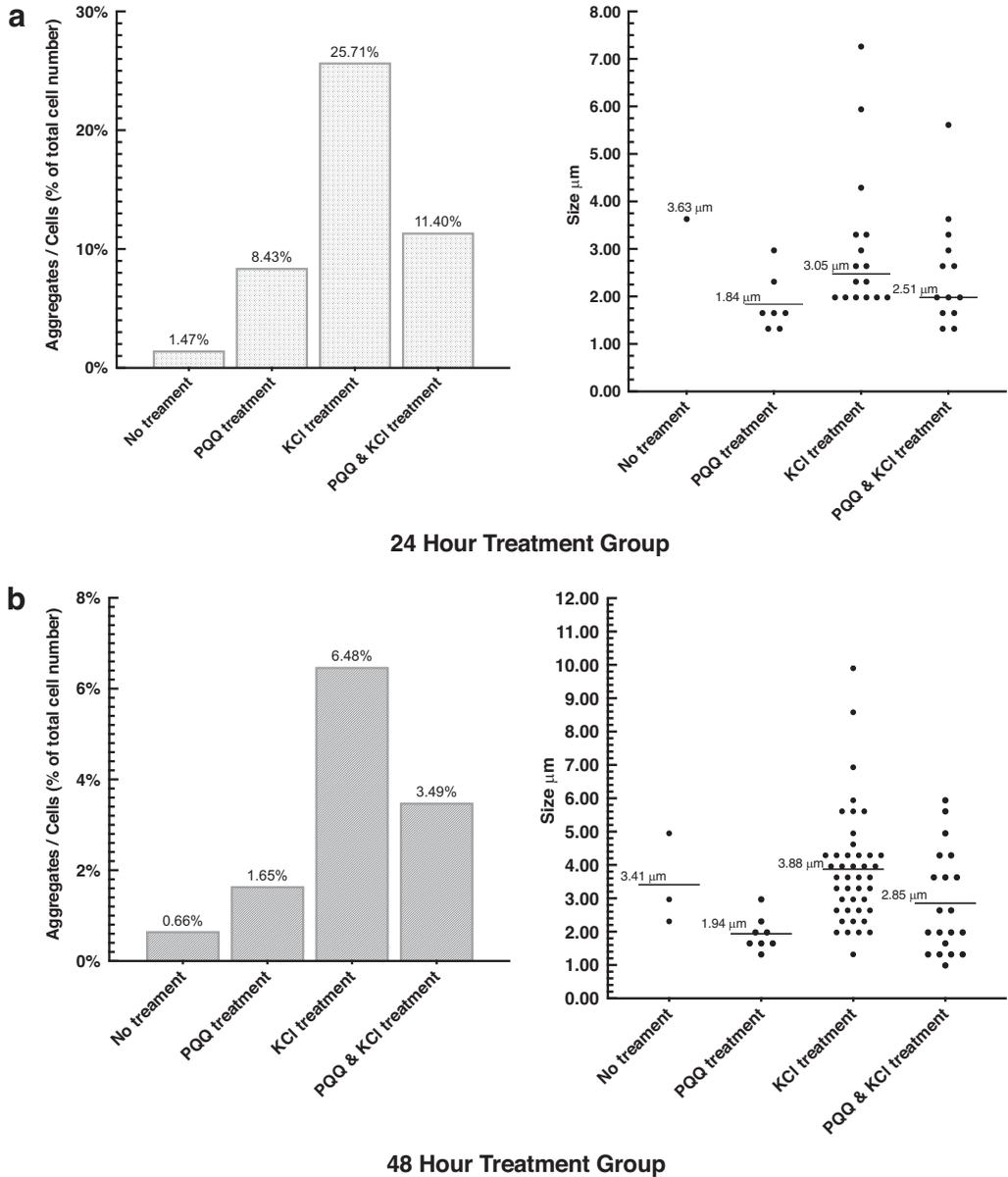
Figure 2. Quantification of alpha-synuclein aggregates shows number and size differences in cells treated with PQQ. Images for both 24 hr (a) and 48 hr (b) were analyzed for inclusion based on sufficient immunofluorescence staining of the alpha-synuclein protein or nucleus and sufficient visibility of cells under confocal microscopy. On identification of images for inclusion, total cells were counted only if DNA staining showed a distinct nucleus, and there was no condensing or blebbing of the cell nucleus. The total number of identified alpha-synuclein aggregates were counted for each sample and divided by the total number of cells in that sample for each cell treatment group per time point. All the identified aggregates of alpha-synuclein proteins were measured in ImageJ v1.51 by tracing around the aggregate's perimeter with a set scale of 1 pixel = 0.21 μm. The mean size of aggregates for each cell treatment group was calculated by totaling the mean size of each aggregate and then dividing by the total number of aggregates. Plots were obtained using Prism GraphPad v9. The line represents the median size of aggregates.

indicating enhanced mitochondria-related function (Harris et al., 2013). Further studies support the fact that PQQ enhances mitochondrial function and improves cognitive performance, increases cerebral blood flow and oxygen use in the prefrontal cortex of the brain, and improves indicators for stress, fatigue, and sleep (Nakano et al., 2012). Interestingly, human trials investigating the consumption of food high in PQQ levels identified improvement in motor skills in Parkinson's disease patients (Rabey et al., 1993). While these studies show PQQ absorption into the blood and improvements in the measured outcomes, they do not address specific tissue, cellular or molecular pathways influenced by the PQQ or PQQ-rich food.

The normal biological function of the alpha-synuclein protein is poorly understood. However, in Parkinson's disease, LB pathology indicates that abnormal aggregation of the protein is a crucial factor in progressive and irreversible loss of dopaminergic cells in both familial and sporadic forms of Parkinson's disease (Levy et al., 2009). Previous in vitro studies have shown PQQ has an anti-aggregation effect on the alpha-synuclein protein in both truncated and full-length forms by reducing the protein's ability to form fibrils and aggregate in addition to reducing its overall toxic influence. However, these studies were not conducted in a Parkinson's disease relevant cellular context (Kim et al., 2010; Kobayashi et al., 2006). In line with these studies, our study demonstrates that the addition of PQQ to preformed aggregates in SH-SY5Y cells not only reduced the number of the aggregates but also indicated the dispersion or reduced size of the aggregates.

Identifying underlying cellular and molecular mechanisms that can clear alpha-synuclein aggregates will allow for greater understanding of Parkinson's disease and allow targeted research. Using in vitro and in vivo studies, a model of these mechanisms has been suggested for PQQ to act as a ligand-like compound for the cell surface receptors that influence key pathway(s) involved in alpha-synuclein posttranslational modification, including ubiquitination, cross-linking, truncations, oxidation (like nitration) and phosphorylation (Burré et al., 2018; Kim et al., 2010; Kobayashi et al., 2006). Extensive research has been conducted on phosphorylated alpha-synuclein and its role in Parkinson's disease with some research in the context of PQQ. Increase of phosphorylated Serine 129 (pSyn-129) alpha-synuclein levels has been noted in Parkinson's disease patients (Kumar et al., 2017), leading to increased aggregation, changes in cell membrane receptor distributions (Canerina-Amaro et al., 2019) and cytotoxicity (Wang et al., 2012). in vitro studies have indicated that PQQ-addition reduces the oligo-merization of the alpha-synuclein aggregates to the levels seen with removal of the Ser129 residue (Kim et al., 2010), indicating that mechanism of action for PQQ may be via interference in phosphorylation of the alpha-synuclein. While the exact cellular pathway is still unclear, it is clear that PQQ can act on the aggregate itself (Kim et al., 2010; Kobayashi et al., 2006) and when enclosed within a cellular context as shown by our study.

To date, Parkinson's disease research has predominately focused on the brain and risk factors that may induce defects in the cellular systems triggering the gradual degeneration and death of dopamine neurons including the potential causal role of alpha-synuclein protein aggregations in cell death. Interestingly, recent evidence points to the gut-brain axis as an essential pathway in Parkinson's disease manifestation, including consistent constipation and gastrointestinal issues that have recently been identified as one of the first symptoms to appear before a patient is diagnosed with Parkinson's disease (Pfeiffer, 2016). Further, recent insights indicate that alpha-synuclein protein may have prion-like characteristics and propagate between cells infecting different regions of the brain (Osterberg et al., 2015) as well as evidence of alpha-synuclein aggregation in enteroendocrine cells (EECs) located in the enteric nervous system of the gastrointestinal tract before they are found in the brain. This suggests that the alpha-synuclein protein's pathological origin may stem from the gastrointestinal tract and spread to the central nervous system via a cell to cell propagation using the vagus nerve (Chandra et al., 2017). Considering the high levels of PQQ in particular food and its use as a supplement, one unexplored avenue of Parkinson's disease research is the influence of food on the gut-brain axis as linked to health and disease. While different models would need to be developed (e.g., enteric neuron cellular model, induction of alpha-synuclein aggregates in the gut cell of an animal model) to further explore the

possibility of disease origin it is important to consider in light of food/diet/supplement as preventative and/or therapeutic intervention.

Extending research into the PQQ mode of action across multiple models is essential to uncover how PQQ impacts the interplay of the development of LBs and the death of dopaminergic neurons. Understanding further the mechanism of action of PQQ on a cellular and molecular level strengthens the clinical trials of diets or supplementation high in this compound, and our findings provide a basis for further cellular, animal, and clinical studies as it specifically shows modifications of intracellular alpha-synuclein aggregates. Finally, targeting the LB formation or therapeutic intervention of formed aggregates using a naturally derived product found in a variety of food while undergoing L-Dopa substitutions may be considered an integrated, evidence-based combinatorial therapeutic strategy for both sporadic and familial Parkinson's disease patients.

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Peer Reviews

Reviewing editor: Dr. Vitor Francisco

University of Coimbra Center for Neuroscience and Cell Biology, Coimbra, Portugal, 3004-504

Minor revisions requested.

Review 1: Pyrroloquinoline Quinone (PQQ) Influences Intracellular Alpha-Synuclein Aggregates

Reviewer: Dr. Francois Cossais [ID]

Date of review: 17 January 2023

Conflict of interest statement. Reviewer declares none.

Comment

Comments to the Author: The authors have addressed the effects of PQQ on alpha-synuclein aggregations using the SHSY5Y cell line. The authors suggest that PQQ reduces the KCL-induced aggregation of alpha-synuclein aggregates. Whereas the manuscript is well written, several experimental issues would need to be addressed before consideration for publication.

– It is not clear how many independent biological experiments were performed. A sufficient number of independent experiments should be done in order to perform statistical analyses in support (or not) of authors' hypotheses. Addition of a vehicle control might be of interest.

– The nature of the observed alpha-synuclein aggregates is poorly characterized. The given reference for alpha-synuclein antibody actually corresponds to an anti histone h3 antibody in the abcam catalog. Do the synuclein aggregate structures also contain phosphorylated alpha-synuclein?

Score Card

Presentation

3.9 /5

Is the article written in clear and proper English? (30%)	5/5
Is the data presented in the most useful manner? (40%)	3/5
Does the paper cite relevant and related articles appropriately? (30%)	4/5

Context

3.0 /5

Does the title suitably represent the article? (25%)	2/5
Does the abstract correctly embody the content of the article? (25%)	3/5
Does the introduction give appropriate context? (25%)	5/5
Is the objective of the experiment clearly defined? (25%)	2/5

Analysis

1.8 /5

Does the discussion adequately interpret the results presented? (40%)	2/5
Is the conclusion consistent with the results and discussion? (40%)	2/5
Are the limitations of the experiment as well as the contributions of the experiment clearly outlined? (20%)	1/5

Review 2: Pyrroloquinoline Quinone (PQQ) Influences Intracellular Alpha-Synuclein Aggregates

Reviewer: Prof. Martin Michaelis ⓘD

University of Kent, School of Biosciences, Canterbury, United Kingdom of Great Britain and Northern Ireland, CT2 7NJ

Date of review: 24 March 2023

Conflict of interest statement. Reviewer declares none.

Comment

Comments to the Author: This is an interesting observation. However, the manuscript is quite wordy and contains a significant amount of information that is not directly relevant. It would benefit from a focus and more detail on the introduction of the model system (and what its use contributes to what is already known) and on the actual results. With regard to the aggregates, it would be interesting to hear whether there is any potential explanation why PQQ single treatment increases their number relative to the untreated control.

- The abbreviation PQQ needs to be introduced in the Abstract.

Score Card

Presentation

3.6 /5

Is the article written in clear and proper English? (30%)	5/5
Is the data presented in the most useful manner? (40%)	3/5
Does the paper cite relevant and related articles appropriately? (30%)	3/5

Context

4.5 /5

Does the title suitably represent the article? (25%)	5/5
Does the abstract correctly embody the content of the article? (25%)	5/5
Does the introduction give appropriate context? (25%)	3/5
Is the objective of the experiment clearly defined? (25%)	5/5

Analysis

3.0 /5

Does the discussion adequately interpret the results presented? (40%)	3/5
Is the conclusion consistent with the results and discussion? (40%)	3/5
Are the limitations of the experiment as well as the contributions of the experiment clearly outlined? (20%)	3/5