1 **Pyrroloquinoline Quinone (PQQ) Influences Intracellular Alpha-Synuclein**

2 **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 improved motor and non-motor functions. Here, we aimed to investigate if and how pyrroloquinoline quinone (PQQ) compound disrupts pre-formed 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 hours. 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.

Background

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 (1). 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 (2). Molecular mechanisms of alpha-synuclein toxicity in the cells include an increase in oxidative stress (2) and damage to the cell membrane structure through the formation of fibrils and pore-like structures (3), 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 (4–6). 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 metabolise 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 (7). However, pharmacological treatments carry significant side effects, risk and diminished response rates as the disease progresses (8) leaving limited treatment options available for Parkinson's disease patients.

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64   Currently, there are limited targeted dietary and/or nutritional interventions integrated into clinical

65   practice to manage Parkinson's disease symptoms in patients. Dietary interventions focus on managing

66   the symptoms of comorbidities such as constipation, weight loss and malnourishment (9). However,

67   several clinical trials and *in vitro* studies identified specific diets and nutrient compounds may assist in

68   improving both motor and non-motor functions. For instance, ketogenic diet trials have shown an

69   improvement in both motor and non-motor symptoms (10,11), supplementation with potent intracellular

70   antioxidants CoQ10 and glutathione, often depleted in Parkinson's disease patients, showed minor

71   improvement in motor symptoms (12–14) and food containing certain nutrient compounds such as

72   carotenoids, vitamin E, sulforaphane, Omega-3, resveratrol, epigallocatechin-3-gallate and caffeine

73   might offer important neuroprotective properties, including the potential to alter the underlying

74   pathological cellular and molecular changes identified in Parkinson's disease (15). Nutrients including

75   selenium, vitamin A, C and E may serve as potent antioxidants protecting the dopaminergic cell from

76   oxidative damage to preserve dopamine producing neurons (8).

77   Interestingly, in a dietary study where participants were given broad beans to consume as part of their

78   daily diet, improvements in motor function and increased levels of endogenous levodopa were observed

79   similar to levels achieved with L-Dopa therapy (16). On further investigation, it was also found that

80   broad beans contain high levels of a nutritional compound Pyrroloquinoline Quinone (PQQ) (17), that

81   has shown to modify alpha-synuclein aggregation *in vitro* (18).

82   PQQ is a water-soluble organic molecule found in varying quantities in vegetables, fruits, and beverages

83   such as tea. PQQ is a vitamin-like compound with combined chemical properties similar to ascorbic

84   acid, vitamin B_2 and vitamin B_6, but with a nutritional requirement similar to folate and biotin (19). In

85   animal studies, PQQ has been demonstrated to be highly absorbable, predominantly in the lower

86   intestine, and non-toxic in both animals and humans, indicating low or no side effects (20). Recent

87   studies have demonstrated that PQQ permeability to the blood-brain barrier is enhanced using

88   esterification processes (21). Importantly for molecular events leading to Parkinson's disease, PQQ was

89   shown to have an anti-aggregation effect on the alpha-synuclein protein *in vitro* (18) and inhibits fibril

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90 formation in both a full length and C-terminal deleted mutant alpha-synuclein *in vitro* (22). However,

91 none of the other alpha-synuclein studies had been conducted in the PD-relevant cellular context.

92 Our study demonstrated that the addition of PQQ to pre-formed alpha-synuclein aggregates can reduce

93 the number and size of these aggregates in a Parkinson's disease SH-SY5Y cellular model. This study

94 is the first to show PQQ aggregation-modifying properties in a Parkinson's disease-specific cellular

95 model. It confirms previous *in vitro* and clinical trial reports establishing this nutritional compound and

96 food containing PQQ as an important player in the regulation of Parkinson's disease symptoms and

97 possible novel treatment approach to Parkinson's disease.

98 **Methods**

99 *Materials*

100 SH-SY5Y neuroblastoma cells (94030304-1VL; Sigma). Hams F-12 nutrient mixture (N48880), Eagles

101 Minimum Essential Medium (EMEM; M2279), Glutamine (GT513), Non-essential amino acids

102 (M7145), Pyrroloquinoline Quinone (D7783-1MG) were purchased from Sigma-Aldrich. Monoclonal

103 antibody to alpha-synuclein (ab27766) was purchased from Abcam, secondary goat anti-mouse Alexa

104 Fluor 488 IgG antibody (A-11001) was from ThermoFisher Scientific and Foetal Bovine Serum (FBS,

105 SFBSF8) from Bovogen. Pyrroloquinoline Quinone (PQQ) was dissolved in 1M sodium hydroxide

106 (NaOH) solution to prepare a stock concentration of 50 mM, and 50µM PQQ was used as the final

107 concentration.

108 *Methods*

109 *Cell Culture*

110 The SH-SY5Y neuroblastoma cells were maintained in the following cell growth media mix - EMEM,

111 Ham's F12 nutrient mixture, 1% glutamine, 1% non-essential amino acids and 15% FBS. Cells were

112 maintained in a humidified incubator at 37°C supplied with 5% CO_2.

113 *Experimental Procedures*

114 **Cell seeding**: 24 hours (h) prior to the experiment, cells were cultured on glass coverslips at 20,000 cells

115 per well of a 12-well multi-well dish. Each experiment contained the following groups in triplicates: No

116 treatment, 50mM KCl treatment, 50μM PQQ treatment, 50mM KCl followed by 50μM PQQ treatment.

117 **PQQ and KCl treatment**: Cells were treated with 50μM PQQ for 24 h, followed by treatment with

118 50mM KCl to the appropriate samples for 60 minutes (min). After 60 min, the PQQ/KCl containing

119 media was decanted and replaced with growth media and incubated further for 24 or 48 h.

120 **Immunofluorescence Assay**: At the 24- and 48-h time points, cells were fixed using 4%

121 paraformaldehyde (prepared in 1 X PBS; PFA/PBS) and permeabilised with 0.1% Triton X-100. Fixed

122 coverslips were stained with primary mouse anti-alpha-synuclein antibody (optimised at 1:50 dilution)

123 and secondary goat anti-mouse Alexa 488 IgG antibody. Hoechst DNA stain diluted in 1X PBS was

124 used for nuclear staining. The coverslips were mounted onto microscopy slides using Fluorescence

125 mounting medium (Dako) and analysed using confocal microscopy.

126 **Confocal Microscopy**: Digitised fluorescent cellular images were collected using a Nikon Eclipse Ti

127 confocal laser-scanning microscope, with a Nikon 60x/1.40 oil immersion objective. Images were

128 captured at an optical thickness of 0.68 μm and an optical resolution of 0.12 μm. An average of 10 – 15

129 images for each treatment group from two independent experiments were captured, resulting in

130 approximately 250 images.

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131 **Image Analysis**: Image analysis was performed in ImageJ v1.51 - images were opened as a hyperstack,

132 split channel colour composite, 16-bit image. Each colour composite image was separated into two

133 multi-level channels (alpha-synuclein and nucleus), and both channels were then merged into one

134 composite image while preserving the original multi-level composite channels. To determine the

135 aggregates' approximate size, a scale was set in ImageJ at 1 pixel to 0.21 μm, and an outline of the

136 aggregates was traced. The ImageJ pre-set function was used to calculate an approximate perimeter size

137 of the aggregate. Aggregate size data and the total number of aggregates was captured for both the 24

138 and 48-h per treatment group and entered into Prism GraphPad v9 to produce a scatter plot and determine

139 differences between groups.

140 Images were discounted if there was either insufficient immunofluorescence staining of the alpha-

141 synuclein or the nucleus, if cells had not shown sufficient growth and proliferation, or if cells were out

142 of focus. Cells were discounted from analyses if the DNA staining did not define a distinct nucleus or

143 there were morphological abnormalities, such as condensing or blebbing of the cell nucleus.

144 **Results**

145 Confocal microscopy revealed two general patterns of alpha-synuclein localisation at both time points

146 investigated - generalised diffusion surrounding the cell nucleus and aggregates predominantly located

147 near the nucleus of the cell (arrows, Figure 1A and 1B).

148 To objectively determine the number of alpha-synuclein aggregates per cell, images were analysed using

149 ImageJ by counting the total number of aggregates and normalising this count to the total number of

150 cells in the image for each sample or by measuring the size of the individual aggregate (Figure 2A and

151 2B, respectively). The highest percentage of aggregates were observed in 50mM KCl only treated

152 samples at both time points, 25.71% and 6.48% compared to 1.47% and 0.66 % in no treatment samples

153 at 24h and 48h, respectively. A three-fold decrease in the number of aggregates was observed in cells

154 treated with PQQ only at both time points, 8.43% and 1.65% at 24h and 48h, respectively. This decrease

155 was two-fold in cells treated with PQQ and stimulated with 50mM KCl compared to control, 11.40%

156 and 3.49% at 24h and 48h, respectively (Figure 2).

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157   The same images were used to determine the average size of all aggregates (Figure 2). A measurable

158   difference in mean aggregate size between the 24 and 48h control and experimental samples were

159   observed. Across all samples, 24h samples contained on average smaller aggregates (Figure 2A). The

160   mean aggregate size in the presence of PQQ only or PQQ + KCl were 1.84 μm and 2.51 μm, respectively,

161   compared to 3.05 μm in the presence of KCl only at 24h. The same trend was evident at 48h with a

162   decrease in mean aggregate size in the presence of PQQ (1.94 μm; PQQ only) and 2.85 μm (PQQ +

163   KCl), compared to KCl only (3.88 μm). In comparison to their controls, in samples incubated with

164   50mM KCl, the mean aggregate size in the presence of PQQ + KCl decreased to 17.7% and 26.5% at

165   24h and 48h, respectively (Figure 2A and 2B).

166   Overall, the cells treated with PQQ without or with KCl consistently demonstrated an overall reduction

167   in the number and mean aggregate size of alpha-synuclein at both 24- and 48-h compared to cells that

168   were not treated with PQQ.

169   **Discussion**

170   In the current study, we used an established Parkinson's disease cellular model, neuroblastoma SH-

171   SY5Y cell line, to demonstrate the reduction in number and size of pre-formed intracellular alpha-

172   synuclein aggregates at 24 and 48h post treatment with PQQ.

173   PQQ was first identified in 1979 as a redox cofactor for bacterial dehydrogenase. Although PQQ is not

174   synthesised in humans, it is found in trace amounts in tissues due to absorption from everyday food,

175   such as broad beans, green peppers, spinach, fermented soybeans, oolong tea, green tea, papaya and

176   kiwi fruit (17). The nutritional requirement for PQQ from a dietary perspective is not defined, but animal

177   studies that modified diets to omit PQQ resulted in impaired growth, immune dysfunction and fertility

178   issues (23).

179   In humans, the nutritional importance of PQQ and the possible clinical applications are increasingly

180   emerging with a primary focus in PQQ supplementation is its potential to have a pharmacological-like

181   role in human disease. In human and animal studies, PQQ is observed to have positive physiological

182   benefits for various diseases including dementia, neurodegenerative disorders, Parkinson's disease,

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183 cardiovascular disease, chronic inflammation, insulin resistance, mood disorders and accelerated aging

184 (24). Animal research models have identified the benefits of PQQ treatments to include overall

185 improvement and even reversal of cognitive impairment, stimulation of nerve growth factors for cell

186 proliferation, protecting cells from oxidative stress and neurotoxicity caused by cerebral ischemia,

187 reduction in neurotoxin-induced injury to the brain (25–27) and protecting nerve cells from beta-amyloid

188 damage in Alzheimer's disease (28). Molecular mechanisms underpinning these observed phenotypes

189 include an increase in mitochondrial energy production, promotion of mitochondrial biogenesis, and

190 AMP kinase activity which serve as a master switch for energy production (29).

191 Human clinical studies using PQQ as a supplement showed an increased presence of the compound in

192 blood and reduced levels of inflammatory markers C-reactive protein (CRP) and interleukin 6 (IL-6)

193 indicating enhanced mitochondria-related function (30). Further studies support the fact that PQQ

194 enhances mitochondrial function and improves cognitive performance, increases cerebral blood flow

195 and oxygen use in the prefrontal cortex of the brain, and improves indicators for stress, fatigue and sleep

196 (19). Interestingly, human trials investigating the consumption of food high in PQQ levels identified

197 improvement in motor skills in Parkinson's disease patients (16). While these studies show PQQ

198 absorption into the blood and improvements in the measured outcomes, they do not address specific

199 tissue, cellular or molecular pathways influenced by the PQQ or PQQ-rich food.

200 The normal biological function of the alpha-synuclein protein is poorly understood. However, in

201 Parkinson's disease, LB pathology indicates that abnormal aggregation of the protein is a crucial factor

202 in progressive and irreversible loss of dopaminergic cells in both familial and sporadic forms of

203 Parkinson's disease (31). Previous *in vitro* studies have shown PQQ has an anti-aggregation effect on

204 the alpha-synuclein protein in both truncated and full-length forms by reducing the protein's ability to

205 form fibrils and aggregate in addition to reducing its overall toxic influence. However, these studies

206 were not conducted in a Parkinson's disease relevant cellular context (18,32). In line with these studies,

207 our study demonstrates that the addition of PQQ to pre-formed aggregates in SH-SY5Y cells not only

208 reduced the number of the aggregates but also indicated the dispersion or reduced size of the aggregates.

209   Identifying underlying cellular and molecular mechanisms that can clear alpha-synuclein aggregates

210   will allow for greater understanding of Parkinson's disease and allow targeted research. Using *in vitro*

211   and *in vivo* studies, a model of these mechanisms has been suggested for PQQ to act as a ligand-like

212   compound for the cell surface receptors that influence key pathway(s) involved in alpha-synuclein post-

213   translational modification, including ubiquitination, cross-linking, truncations, oxidation (like nitration)

214   and phosphorylation (18,32,33). Extensive research has been conducted in phosphorylated alpha-

215   synuclein and its role in Parkinson's disease with some research in the context of PQQ. Increase of

216   phosphorylated Serine 129 (pSyn-129) alpha-synuclein levels have been noted in Parkinson's disease

217   patients (34), leading to increased aggregation, changes in cell membrane receptor distributions (35)

218   and cytotoxicity (36). *In vitro* studies have indicated that PQQ-addition reduces the oligomerisation of

219   the alpha-synuclein aggregates to the levels seen with removal of the Ser129 residue (32), indicating

220   that mechanism of action for PQQ may be via interference in phosphorylation of the alpha-synuclein.

221   While the exact cellular pathway is still unclear, it is clear that PQQ can act on the aggregate itself

222   (18,32) and when enclosed within a cellular context as shown by our study.

223   To date, Parkinson's disease research has predominately focused on the brain and risks factors that may

224   induce defects in the cellular systems triggering the gradual degeneration and death of dopamine neurons

225   including the potential causal role of alpha-synuclein protein aggregations in cell death. Interestingly,

226   recent evidence points to the gut-brain axis as an essential pathway in Parkinson's disease manifestation,

227   including consistent constipation and gastrointestinal issues that have recently been identified as one of

228   the first symptoms to appear before a patient is diagnosed with Parkinson's disease (37). Further, recent

229   insights indicate that alpha-synuclein protein may have prion-like characteristics and propagate between

230   cells infecting different regions of the brain (38) as well as evidence of alpha-synuclein aggregation in

231   enteroendocrine cells (EECs) located in the enteric nervous system of the gastrointestinal tract before

232   they are found in the brain. This suggests that the alpha-synuclein protein's pathological origin may stem

233   from the gastrointestinal tract and spread to the central nervous system via a cell to cell propagation

234   using the vagus nerve (39). Considering the high levels of PQQ in particular food and its use as a

235   supplement, one unexplored avenue of Parkinson's disease research is the influence of food on the gut-

236   brain axis as linked to health and disease. While different models would need to be developed (e.g.

237   enteric neuron cellular model, induction of alpha-synuclein aggregates in the gut cell of an animal

238   model) to further explore the possibility of disease origin it is important to consider in light of

239   food/diet/supplement as preventative and/or therapeutic intervention.

240   Extending further research into the PQQ mode of action across multiple models is essential to uncover

241   how PQQ impacts the interplay of the development of LBs and the death of dopaminergic neurons.

242   Understanding further the mechanism of action of PQQ on a cellular and molecular level strengthens

243   the clinical trials of diets or supplementation high in this compound, and our findings provide a basis

244   for further cellular, animal and clinical studies as is specifically shows modifications of intracellular

245   alpha-synuclein aggregates. Finally, targeting of the LB formation or therapeutic intervention of formed

246   aggregates using a naturally derived product found in a variety of food while undergoing L-Dopa

247   substitutions may be considered an integrated, evidence-based combinatorial therapeutic strategy for

248   both sporadic and familial Parkinson's disease patients.

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369 **Figure 1: Images of alpha-synuclein aggregates show morphology change in the presence of**

370 **PQQ.**
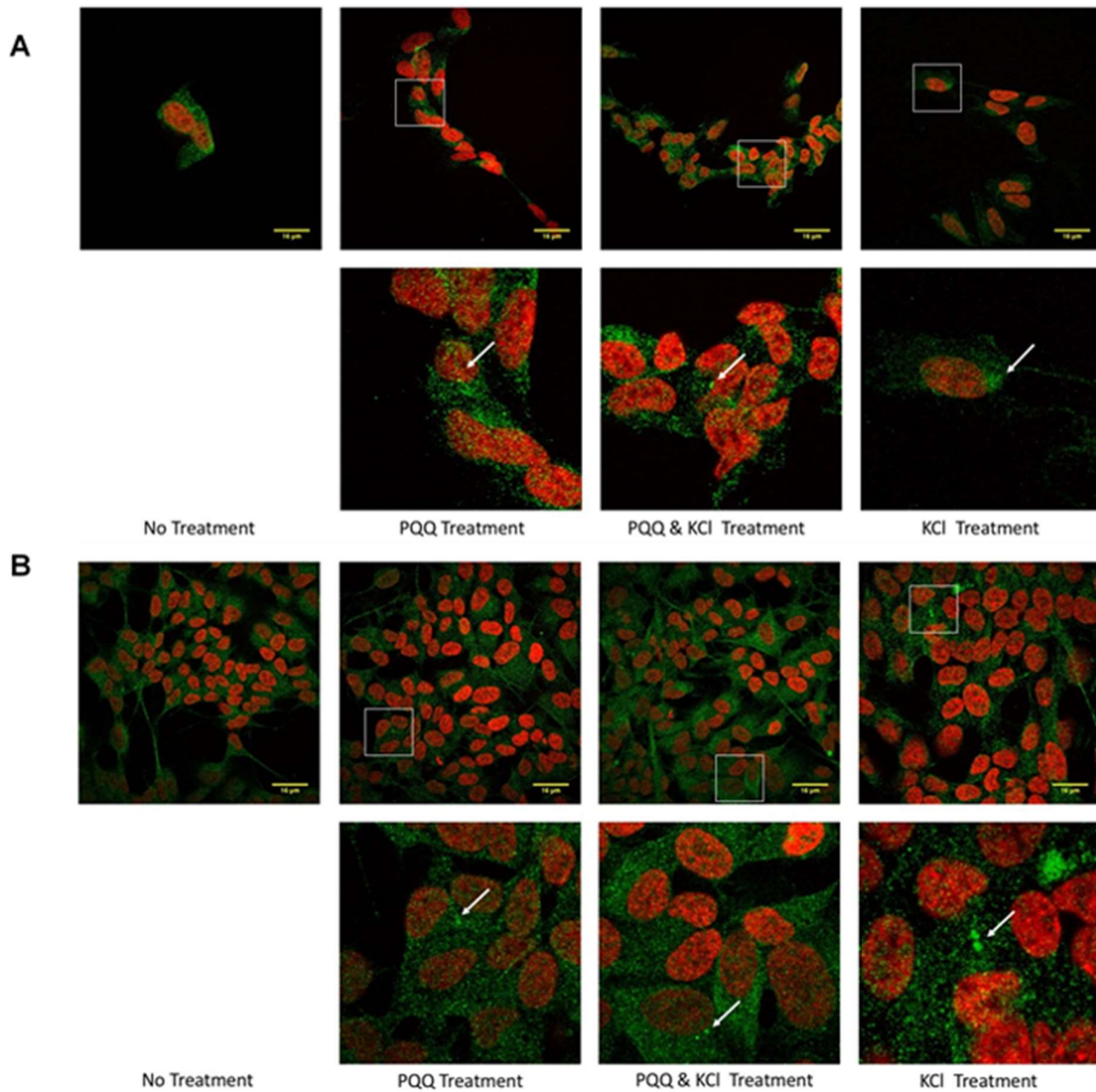
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372 SH-SY5Y cells were incubated in the presence or absence of 50mM PQQ for 1 hour and then for a
373 further hour with 50mM KCl treatment as appropriate for the sample to induce alpha-synuclein
374 aggregation. After KCl treatment the cells were washed into full cell media containing 50 mM PQQ
375 ("PQQ treatment "and "PQQ & KCl treatment") or full cell media only ("no treatment" and "KCl
376 treatment") for 24 h (A) or 48 h (B). The cells were fixed and then stained using anti-alpha-synuclein
377 antibody and secondary goat anti-mouse Alexa Fluor 488 IgG antibody. Hoechst 3342 diluted in 1X
378 PBS was used for nuclear staining (shown in red). The images were taken with a Nikon Ti Eclipse
379 confocal laser-scanning microscope and NIS Elements AR software. Scale bar: 16 μm.

Figure 2: Quantification of alpha-synuclein aggregates shows number and size difference in cells treated with PQQ.
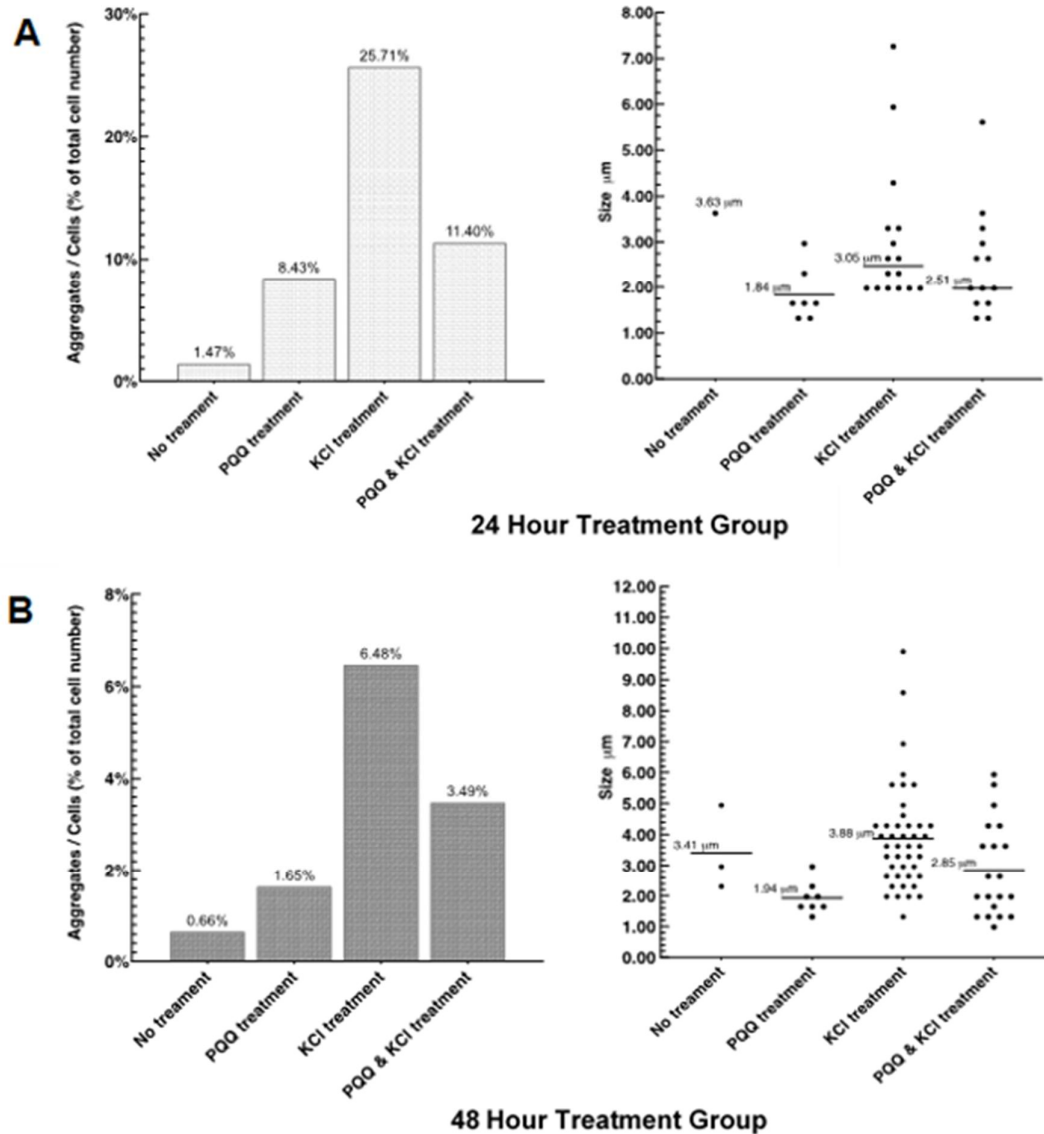
Images for both 24 h (A) and 48 h (B) were analysed for inclusion based on sufficient immunofluorescence staining of the alpha-synuclein protein or nucleus and sufficient visibility of cells under confocal microscopy. On identification of images for inclusion, total cells were counted only if DNA staining 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 totalling 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.