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Antioxidative and neuroprotective activities of peanut sprout extracts against oxidative stress in SK-N-SH cells



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ABSTRACT

Objective: To evaluate the protective effect of peanut sprout extract (PSE) against paraquat (PQ) induced SK-N-SH cells.**Methods:** Three groups of cells were used in the experiment, together with a fourth, control group. One group was treated with PQ, the second group was treated with PSE, and the third group was pre-treated with PSE. The control group was untreated. Cell viability and toxicity were detected by MTT assay, cellular reactive oxygen species (ROS) was detected by Muse Cell Analyzer, quantitative RT-PCR was applied to investigate the expression of SIRT1 and α -synuclein genes, and A β 42 was detected by western blot.**Results:** The 50% effective concentration of PQ was 0.75 mmol/L. PSE had no significant cytotoxicity at a concentration of 1.5 mg/mL. In the group of cells pre-treated with PSE, cell death was significantly inhibited. In the PQ treated group, PQ was increased in the intracellular ROS in the cells. Intracellular ROS was significantly decreased in the cells treated with PSE and also those pre-treated with PSE. PSE significantly downregulated the expression of SIRT1 and α -syn genes, and it was found that PQ significantly increased β -amyloid 42 levels whereas this action was inhibited by PSE.**Conclusions:** PSE has neuroprotective activities against oxidative stress in SK-N-SH cells induced by PQ, suggesting that PSE is a highly promising agent in the prevention of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease.

1. Introduction

Neurodegeneration is the progressive loss of structure or function of neurons, including the death of neurons. Many neurodegenerative diseases including Alzheimer's disease (AD) and Parkinson's disease (PD) occur as a result of neurodegenerative processes [1]. AD has been defined by the presence of

extracellular amyloid- β (A β) containing plaques and cytoplasmic neurofibrillary tangles (NFT) consisting of abnormal microtubule associated protein tau. These proteinaceous aggregates are accompanied by synapse loss and neuronal cell death, which are thought to subserve the clinical syndrome of progressive cognitive impairment in AD [2,3]. PD is associated with progressive loss of dopaminergic neurons in the substantia nigra, as well as with more widespread neuronal changes that cause complex and variable motor and non-motor symptoms [4]. It is found that α -synuclein is a major component of Lewy bodies and Lewy neurites, the pathological hallmarks of PD, indicating its role in PD pathogenesis [5]. Environmental factors are important contributory factors in neurodegenerative disease [6].

Paraquat (PQ) (1,1-dimethyl-4,4'-bipyridinium dichloride) is a widely used herbicide. It has been suggested that PQ might be an environmental factor contributing to neurodegenerative disorder

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[7–9]. Studies using animal models have also indicated the neurotoxicity of PQ in nigrostriatal dopaminergic cells [10]. PQ reproduces the cardinal PD pathologies such as loss of dopaminergic neurons [11] and protein aggregation in dopaminergic neurons [12] as well as other pathologies that include oxidative stress [13], proteasome dysfunction [14], and mitochondrial dysfunction [15]. In addition, PQ in the presence of oxygen generates the superoxide radical [16,17], hydroxyl radical, and hydrogen peroxide (H_2O_2) leading to deleterious effects on cell function [8,18,19]. H_2O_2 induces SIRT1 overexpression [20]. SIRT1 has a dual effect on FOXO3 function by increasing FOXO3's ability to induce cell cycle arrest and to resist oxidative stress [21].

In recent years, nature has been a continuous source of pharmacologically active molecules and medicinal herbs [22]. Peanut sprouts have been noted for their antioxidant properties and the germinated peanut kernels have been used in the diet as a health food for several centuries. It has been reported that peanut sprouts are rich in flavonoids and phenolic compounds which may contribute to disease prevention and have health promoting properties [23,24]. They exhibit many biological functions such as anti-inflammatory activity attributed to inhibition of cyclooxygenase, estrogenic activity, and antiplatelet activity [25–27]. Moreover, it has been reported that flavonoids and phenolic compounds have a beneficial effect in the treatment of ischemia [28] and neurodegenerative disease [29]. Therefore, the purpose of the present study was to investigate the protective and antioxidative effects of peanut sprout extract (PSE) on PQ-induced SK-N-SH.

2. Materials and methods

2.1. Materials

Minimum essential medium, fetal bovine serum, 0.25% trypsin–ethylenediaminetetraacetic acid solution and 1% penicillin–streptomycin solution were purchased from Gibco (Invitrogen, Grand Island, NY, USA). Dimethyl sulfoxide and methyl viologen dichloride hydrate were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). MTT was purchased from Bio Basic Inc. (Markham, Canada). Muse[®] Oxidative Stress Kit was purchased from Merck Millipore Corporation (Germany). RiboZol RNA extraction reagent was purchased from Amresco (USA). DNase I and 50 mmol/L ethylenediaminetetraacetic acid were purchased from Fermentas (Thermo Fisher Scientific, USA). Tetro reverse transcriptase and SensiFAST[™] SYBR[®] Kits were purchased from Biorline (Meridian Life Science, USA). Antibody for western blot was purchased from Merck Millipore Corporation (Germany) and 3,3'-diaminobenzidine reagent was purchased from Bio Basic Canada Inc.

2.2. Germination of peanut kernels and PSE

Mature peanut kernels (*Arachis hypogaea* cv. Tainan 9) were soaked in normal saline for 3 h, and then washed with sterile water three times and then soaked in normal saline for 30 min. The kernels were placed on a plastic net tray and germinated in a growth chamber for 3 days in the dark. After 1 day of incubation,

the ungerminated kernels were discarded. After 3 days, the peanut sprouts were weighed and dried for 72 h at 60 °C.

The sprouted peanuts were ground and 100 g of the ground peanut powder was mixed with 100 mL of hexane and incubated overnight on a hot plate stirrer. The mixture was then passed through filter paper and 100 mL of 80% ethanol was added and the mixture was incubated overnight on a hot plate stirrer. The mixture was filtered with filter paper prior to rotary evaporation at 50 °C and 50 mmHg. The PSE was dried at 50 °C prior to use.

2.3. Cell culture

Human neuroblastoma cells (SK-N-SH) were obtained from the American Type Culture Collection (Manassas, VA, USA). These cells were cultured in 10% (v/v) fetal bovine serum and 1% penicillin–streptomycin solution and maintained at 37 °C in humidified incubator with 5% CO_2 .

2.4. Cell viability/cytotoxicity assay

An MTT reduction assay was used to assess the viability of the cells. The cells were seeded in 96-well plate at a density of 3×10^4 cells per well and incubated overnight at 37 °C in 5% CO_2 . The cells were then treated with PQ (0–1 mmol/L) and PSE (0.25–1.5 mg/mL) for 48 h. In one group, referred to as the prevention group, the cells were pre-treated with PSE for 4 h and then further treated with PQ (0.75 mmol/L) for 48 h. A solution of 1 mg/mL MTT was added to each well and the cells were further incubated for 4 h at 37 °C, 5% CO_2 . The liquid in the wells was then removed. The reaction with the MTT had produced purple MTT formazan crystals which were then dissolved in dimethyl sulfoxide. The product was measured by a microplate reader at 540 nm. The percentage of cell viability was normalized to the control group.

2.5. Intracellular reactive oxygen species (ROS) determination

The cells undergoing oxidative stress defined by the presence of ROS, namely, superoxide, were determined by Muse[®] Oxidative Stress Kit. Briefly, after culturing and treatment, the cells were re-suspended at a concentration of 1×10^6 cells per mL in 1× assay buffer (Muse[®] Oxidative Stress Kit). After that, the samples were incubated for 30 min at 37 °C and then the ROS positive cells were examined using the Muse[®] Cell Analyzer.

2.6. RNA analysis

Total RNA was extracted from the cells produced in the cell culturing activity, with RiboZol RNA extraction reagent. To discard genomic DNA, the total RNA (500 ng) was treated with DNase I. First-strand cDNAs were synthesized from the total RNA (250 ng) by Tetro reverse transcriptase and oligo primer were incubated at 45 °C for 30 min. This reaction was terminated by incubating the treated total RNA at 85 °C for 5 min. The synthesized cDNAs were further utilized for quantitative PCR analysis.

The gene expression levels were determined by quantitative PCR using LightCycler[®] 96 (Roche Diagnostics) and

SensiFAST™ SYBR® Kits. The sequences of the primers used in this study were as follows: SIRT1 (forward: 5'-TCAGTGGCTG-GAACAGTGAG-3'/reverse: 5'-AGCGCCATGGAAAATGT-AAC-3'), α -synuclein (forward: 5'-GTGCTCAGTTCCA ATGTGCC-3'/reverse: 5'-TGGGGGCAGGTACAGATACT-3') and β -actin (forward: 5-CC ACCATGTACCCTGGCATT-3'/reverse: 5-CCAACCTCGTCATACTCCTGC-3'). The data were expressed as the mean \pm SEM from three independent experiments. Transcription levels of all genes were normalized to the level of the β -actin gene used as the internal control.

2.7. Western blotting

After culture and treatment, the cells were washed with phosphate buffered saline and then suspended in 100 μ L of radioimmunoprecipitation assay lysis buffer. The protein concentration was determined using a bicinchoninic acid protein assay. Proteins were separated using 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrically transferred to a polyvinylidene fluoride transfer membrane. After the membrane was blocked with 5% skim milk, target proteins were immune-detected using specific antibodies. Primary antibodies were composed of anti- β -actin and anti-beta-amyloid 1–42. Horseradish peroxidase-conjugated anti-rabbit immunoglobulin G was applied as the secondary antibody, and bands were detected using chromogenic detection of horseradish peroxidase activity based on the action of 3,3'-diaminobenzidine in western blot methods.

2.8. Statistical analysis

All results were expressed as the mean \pm SEM. The data were analyzed by One-way ANOVA using SPSS, version 17.0. Differences were considered to be significant ($P < 0.05$).

3. Results

3.1. PQ induced cytotoxicity in SK-N-SH cells

SK-N-SH cells were treated with PQ at various concentrations (0, 0.25, 0.5, 0.75 and 1 mmol/L) for 48 h. Cell viability was decreased in a concentration-dependent manner by treatment with PQ. The presence of 0.5–1 mmol/L PQ significantly reduced cell viability ($P < 0.05$) as compared with 0 mmol/L PQ (Figure 1). This result indicated that PQ enhanced the death of the SK-N-SH cells.

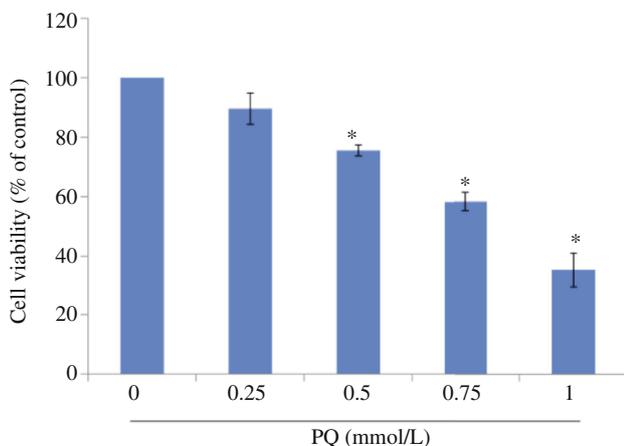


Figure 1. Effect of PQ on the cell viability of SK-N-SH cells. *: $P < 0.05$ as compared with 0 mmol/L.

3.2. PSE induced cytotoxicity in SK-N-SH cells

PSE promoted cell viability at concentrations of 0.25, 0.5, 1, and 1.5 mg/mL up to about 100.89% \pm 2.00%, 104.57% \pm 1.67%, 116.29% \pm 7.98%, and 126.29% \pm 5.35% respectively. The 1.5 mg/mL PSE significantly enhanced cell viability up to about 26% as compared with the control.

3.3. Effect of PSE on SK-N-SH cells induced by PQ

In order to determine the protective effect of PSE on SK-N-SH cells induced by PQ, the results showed that PSE significantly promoted cell viability at concentrations of 1 and 1.5 mg/mL up to about 16.5% and 23.6% as compared to PQ, respectively (Figure 2). This result indicated that PSE had a protective effect on PQ-induced SK-N-SH.

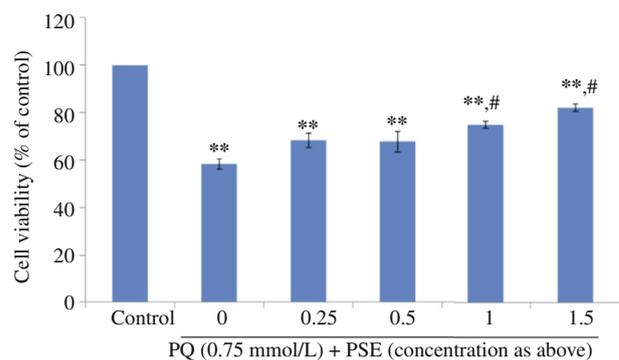


Figure 2. Neuroprotective activity of PSE in SK-N-SH cells.

** : $P < 0.005$ as compared with the control; #: $P < 0.005$ as compared with 0.75 mmol/L PQ.

3.4. Effect of PSE on intracellular ROS in SK-N-SH cells

A significant increase in intracellular ROS was found in the SK-N-SH cells after they were treated with 0.75 mmol/L when compared to the control (Figure 3). However, the groups treated with PSE and the group pre-treated with PSE showed significant decreases in intracellular ROS when compared to 0.75 mmol/L PQ (Figure 3).

3.5. Effect of PSE on SIRT1 and α -synuclein expression

After the cells had been treated with 1 mg/mL for 4 h, they were further treated with 0.75 mmol/L PQ for 48 h. The data showed that SIRT1 and α -synuclein mRNA level in the PQ-treated cells significantly increased when compared with control group. Treatment with PSE only and PSE pre-treatment significantly decreased level of SIRT1 and α -synuclein mRNA expression by 1.30, 2.12 and 0.50, 1.07, respectively (Figures 4 and 5).

3.6. Effect of PSE on A β 42 protein expression

The protein level in the PQ-treated cells increased significantly as compared to the control group. Treatment with PSE only showed result similar to control group. PSE pre-treatment significantly decreased the level of A β 42 about 0.27 as compared with 0.75 mmol/L PQ (Figure 6).

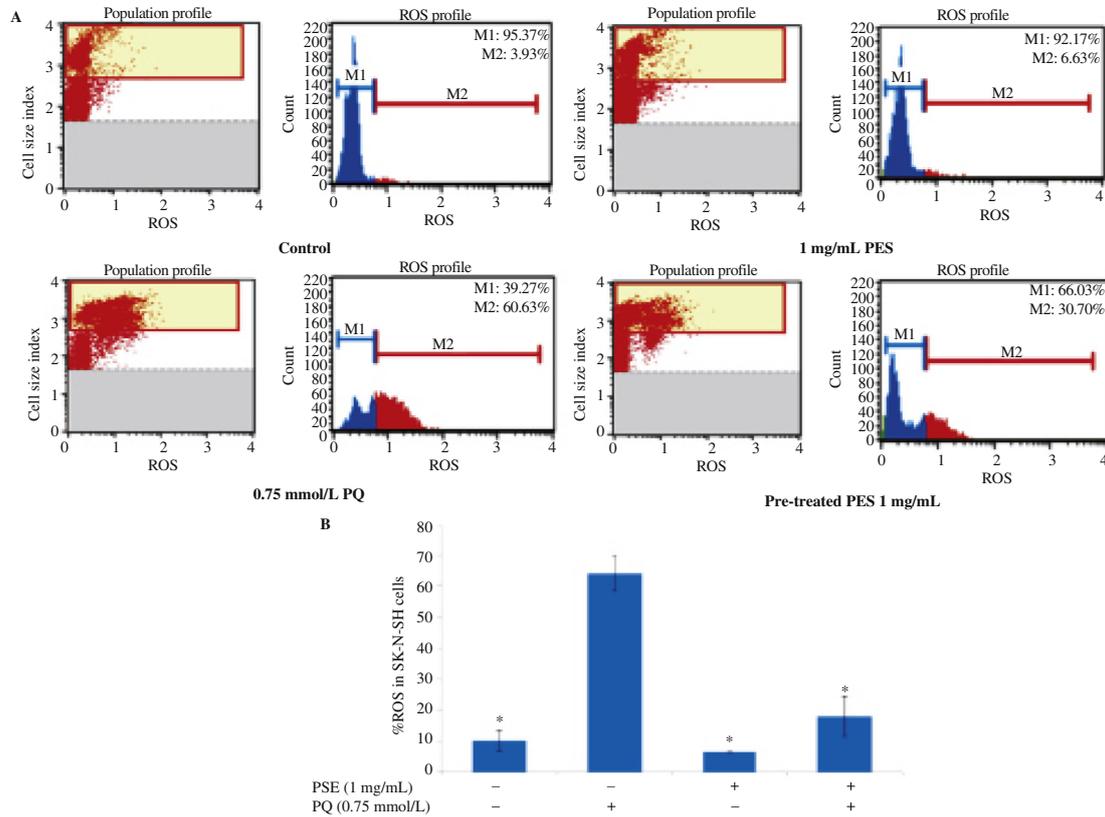


Figure 3. Effect of PSE on intracellular ROS in SK-N-SH cells.

A: The typical picture of ROS detected by Muse Cell Analyzer; B: Percentages of ROS. *: $P < 0.05$ as compared with 0.75 mmol/L. M1: Negative intracellular ROS; M2: Positive intracellular ROS.

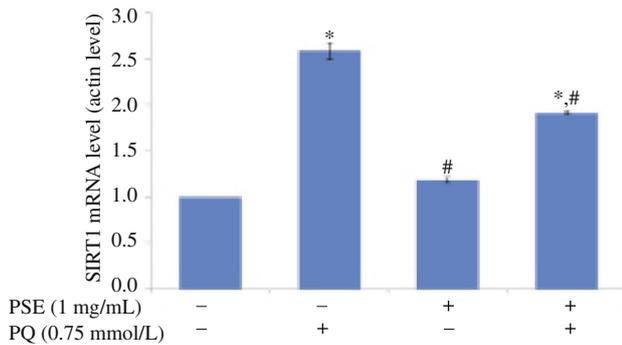


Figure 4. Expression of SIRT1 in SK-N-SH cells, relative SIRT1 mRNA levels calculated by the $2^{-\Delta\Delta Ct}$, normalized to β -actin mRNA level.

#: $P < 0.05$ as compared with 0.75 mmol/L; *: $P < 0.05$ as compared with control.

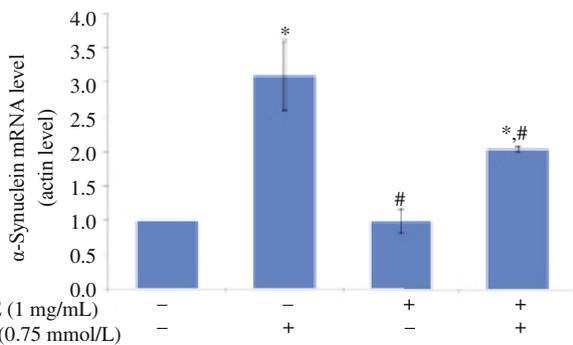


Figure 5. Expression of α -synuclein in SK-N-SH cells, relative α -synuclein mRNA levels calculated by the $2^{-\Delta\Delta Ct}$, normalized to β -actin mRNA level.

#: $P < 0.05$ as compared with 0.75 mmol/L; *: $P < 0.05$ as compared with control.

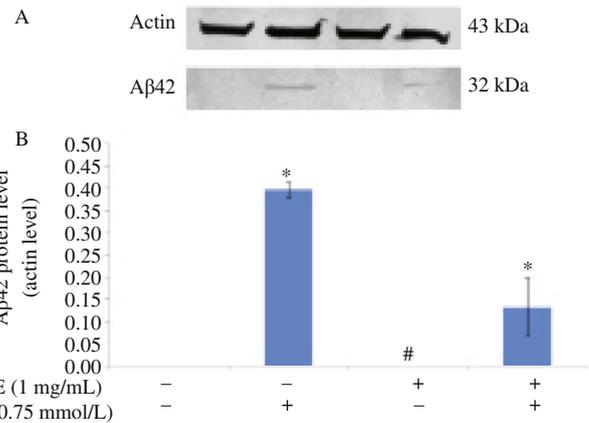


Figure 6. Effect of PSE on protein level of Aβ42.

A: Representative photograph of Aβ42; B: Protein level of Aβ42. The level of protein was quantified by ImageJ and normalized to actin level. *: $P < 0.05$ as compared with control; #: $P < 0.05$ as compared with 0.75 mmol/L.

4. Discussion

In this study, we observed a protective effect of PSE against PQ-induced oxidative stress in SK-N-SH. It has been reported that PSE protects against ultraviolet B-induced oxidative stress by the activation of Nrf2 and the upregulation of Nrf2-related antioxidants. PSE plays an important role in enhanced resveratrol biosynthesis [30,31]. PSE contains more polyphenols, especially resveratrol than peanut extract [23]. Antioxidants from nutritional sources can protect against the death of neuronal

cells in AD and also modulate A β -induced oxidative stress [32]. Thus, it has been postulated that PSE has antioxidant properties that can act to protect against oxidative stress-induced cell death.

We found that PQ induced the generation of ROS in SK-N-SH cells. This finding is consistent with PQ-induced oxidative stress in brain mitochondria, via increased ROS such as H₂O₂ and superoxide anion [15,33]. However, Day *et al.* found that PQ generated superoxide through redox cycling with intracellular diaphorases and molecular oxygen [34].

SIRT1 has been implicated in the activation of anti-apoptotic, anti-inflammatory, anti-stress responses, and aggregation of proteins involved in neurodegenerative disorders [35]. SIRT1 is regulated through p53, nuclear factor- κ B, MyoD, PGC-1, and FOXO3 [36]. Our study showed that PQ-treated SK-N-SH cells overexpressed SIRT1 mRNA, which is in agreement with Castello *et al.* [15] where it was reported that PQ induced ROS generation. Our results are also consistent with a previous report that ROS affected the high expression of SIRT1 [20]. PQ may trigger mechanisms of antioxidative defenses through SIRT1 overexpression to rescue PQ-treated SK-N-SH cells. PSE, which possesses antioxidant properties, can inhibit PQ-induced ROS production leading to decreased expression of SIRT1.

PQ has been found to cause neurodegenerative diseases and induce lipid peroxidation and consequential cell death of dopaminergic neurons that are observed in the onset of the parkinsonian syndrome [8]. Further, α -synuclein is the major protein component of Lewy bodies, a cardinal pathological feature of the degenerating parkinsonian brain. PQ induces the conformational change in the α -synuclein structure and significantly accelerates the formation rate of α -synuclein fibrils *in vitro* [37,38], and α -synuclein has been found to induce mitochondrial dysfunction and oxidative stress [39]. These actions of PQ have been implicated in the formation of aggregated α -synuclein [40]. In our study, we found that PQ not only possesses a potent toxic effect in SK-N-SH cells but also induces overexpression of α -synuclein genes. Interestingly, PSE can downregulate the α -synuclein gene, which has been demonstrated by Caruana *et al.*, who found that the inhibition and disaggregation of α -synuclein oligomers are a result of natural polyphenolic compounds [41]. Our study demonstrated that PSE can downregulate α -synuclein and protect against cell death.

The neuropathology of AD is characterized by the presence of extracellular neuritic plaques (amyloid plaques), intracellular NFT and loss of cholinergic neurons in basal forebrain [42,43]. Oxidative stress leads to the formation of amyloid plaques and NFT [44]. In our study, PQ was shown to increase A β 42 protein levels in SK-N-SH cells. Chauhan and Chauhan reported that PQ induced production of ROS which could induce β - and γ -secretases leading to an increase in A β production from amyloid precursor protein (APP) [45]. Our results showed that A β 42 protein was in oligomer, protofibril, and eventually amyloid fibril aggregates. Oligomer and protofibril are putative toxic species that drive neuronal dysfunction [46,47]. Resveratrol in PSE expressed antioxidant properties and also downregulated the A β 42 protein [23,31]. Resveratrol has also been shown to be able to prevent and alleviate the numerous neurodegenerative disorders and age-related neurological decline [48]. Therefore, it can be postulated that PSE downregulates A β 42 protein and decreases cell death.

The overall conclusion of this study, exposure to PQ leads to oxidative stress in SK-N-SH cells. PSE, which possesses antioxidant properties, can protect against cell death and inhibits

PQ-induced ROS production leading to decrease in progressive neurodegeneration. SIRT1 may function through FOXO3 to affect the antioxidant system. ROS may be relevant in the activation of β - and γ -secretases to increase A β production from APP. A β and APP may also directly induce the production of ROS. In addition, ROS activates α -synuclein aggregation, associating with induction of ROS production in neurodegenerative diseases.

Conflict of interest statement

We declare that we have no conflict of interest.

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