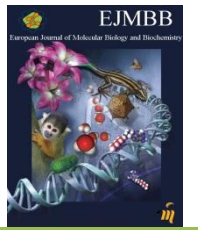
e - ISSN - 2348-2206
Print ISSN - 2348-2192

European Journal of Molecular Biology and Biochemistry

Journal homepage: www.mcmed.us/journal/ejmbb



PROTECTIVE EFFECT OF SESAMOL ON ROTENONE-INDUCED C6 CELL LINE AND RAT BRAIN

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Article Info
Received 08/08/2017
Revised 16/08/2017
Accepted 22/08/2017

Key words:-
Parkinson's disease, Rotenone, Sesamol, C6 cell line, Brain.

ABSTRACT
To investigate the protective effect of sesamol on rotenone-induced C6 cell line and rat brain. C6 cells were incubated with rotenone (20 µM) and sesamol at different concentrations (10-100 µM). Cell viability was determined by MTT assay. The reactive oxygen species, mitochondrial membrane potential and nuclear morphology were determined by dichlorofluorescein diacetate, rhodamine 123 and 4', 6- diamidino -2-phenylindole, respectively. Experiments were carried out for 60 days in male Wistar albino rats (n= 6: I- vehicle control, II- rotenone (3 mg/kg.B.wt intraperitoneal), III- rotenone + sesamol (50 mg/kg.B.wt intraperitoneal), IV- rotenone + sesamol + L-DOPA (10 mg/kg.B.wt oral), V- rotenone + L-DOPA). Histopathology of the brain was assessed. Sesamol significantly increased the cell viability in rotenone-induced C6 cells. Sesamol reduced rotenone-induced reactive oxygen species generation, mitochondrial membrane potential impairment and nuclear damage. Histopathological evidences revealed that sesamol attenuated the injury caused by rotenone in the brain. The results obtained strongly indicate the promising role of sesamol against rotenone in both C6 cells and rat brain. Hence, sesamol can be helpful in the management of Parkinson's disease treatment.

INTRODUCTION

Oxidative stress and inflammation are attributed to neurodegenerative diseases [1,2]. Inflammatory cells release a huge amount of reactive species at the site of inflammation leading to oxidative stress [3]. The brain is vulnerable to oxidative stress due to the exposure towards reactive oxygen species (ROS) and reactive nitrogen species (RNS) [4]. Parkinson's disease (PD) is related to neuroinflammation, in which the hallmarks are the occurrence of reactive astrocytes and activated microglia in the parenchyma of the central nervous system [5]. The interactions between glial cells and neurons have an intense interest for neuroscientists [6-9]. Kimelberg and Nedergaard [10] reported that during brain damage, astrocytic functions become impaired and their impact on neuronal cells lead to neurodegenerative diseases.

Astrocytes are the supreme and frequently occurring glial cells in the central nervous system [11]. Astrocytes are involved in numerous regulatory pathways of the brain environment, mostly regarding neurotransmitter systems, ionic homeostasis, synaptic transmission, metabolic support, free-radical scavenging, maintenance of the blood-brain barrier and immune function [12-14]. Astrocytes express numerous receptors that allow them to respond to numerous neuroactive compounds, including neurotransmitters, neuropeptides, growth factors, cytokines, small molecules and toxins [15]. In brain, neurons cannot survive without communication with astrocytes [16,17] and when astrocytes fail, there will be failure of neuronal survival. During brain damage, astrocytes become transiently or permanently impaired and the impact of neuronal cells may lead to the pathogenesis of PD [6,18,19]. Astrocytes function can critically

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influence neuronal survival and has been established that activated astrocytes might take part in the inflammatory responses in the pathogenesis of dopaminergic neuronal degeneration [20]. Astrocytes activation shows an increased expression of glial fibrillary acidic protein (GFAP), enlarged cell body and projections in the immune-mediated diseases [21]. Thus, astrocytes are involved in both exacerbation of impairment and in neuroprotective processes that are vital for neuronal existence. Human epidemiological studies show that exposure to pesticides, herbicides and heavy metals increases the risk of PD [22,23].

Numerous studies carried out with rotenone (ROT) are associated with oxidative damage of neurons. Greenamyre, *et al*. [24] reported that ROT is a naturally present isoflavonoid produced by the tropical legumes, it is extremely hydrophobic and penetrates through the biological membranes, inhibits complex I and cause ATP (adenosine triphosphate) exhaustion. Several lines of evidence have shown that administration of ROT [25,26] reproduces key features of PD, including degeneration of dopaminergic neurons [27], activation of glial cells [28], movement disorders [29] and defects in mitochondrial complex I [30].

Sesamol (SES) (5-hydroxyl-1,3-benzodioxole or 3,4-methylenedioxyphenol) is the chief component of sesame oil which is potentially anti-oxidative [31]. It is a well- known antioxidant due to the presence of benzodioxole group in its ring known to scavenge hydroxyl radical [32]. SES reduces oxidative stress and prevents organ injury [33]. Several studies have reported that SES is said to have neuroprotective effects [34-36]. Recently, an emergent body of suggestion revealed that the malfunctioning of astrocytes may lead to various neurodegenerative diseases like Alzhemier's disease, multiple sclerosis and PD. Hence, we have concentrated on neuronal supportive cells like astrocytes which have not been studied in greater extent with the aspects of ROT-induced damage. Therefore, the current study examined the glial responses using C6 cells. C6 cells are broadly used astrocyte like cell line [37]. Natural extract products are recently involved for prevention and management of neurodegenerative diseases.

In our earlier study, SES an antioxidant protected the SH-SY5Y neuronal cells against ROT-induced damage [38]. Without understanding the mechanisms of cell death in PD, it is difficult to define targets for treatment. Hence, by *in vitro* method, the initial step of drug discovery and development has been carried out in this study. Further *in vivo*, using animal model is being attempted to identify the potential neuroprotective agent.

MATERIALS AND METHODS

Rotenone, sesamol, L-DOPA (L-dihydroxy phenylalanine), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), dichlorofluorescein diacetate (DCFH-DA), rhodamine 123, 4',6- diamidino-2-phenylindole (DAPI), dimethyl sulphoxide (DMSO), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Hematoxylin and eosin were purchased from Sigma-Aldrich (St.Louis, Missouri, USA).

Cell culture and Treatments

C6 cells were obtained from the National Centre for Cell Science (NCCS), Pune, India. Late passage cells [39] were seeded in flask and cultured in DMEM with 10% FBS, L-glutamine, 100U/mL penicillin/streptomycin in 95% humidified air and 5% CO_2 incubator at 37°C. Cells were detached from culture flask and seeded with density of $1x10^5$ cells/well. After cells reached confluence, the medium was removed and cells were incubated in DMEM without serum. ROT and SES were freshly prepared in DMSO and saline respectively and induced in cells. The final concentration of DMSO in the medium was always less than 0.1% and it has revealed no effects on cell viability. The effective dose of SES was used to identify its potential effect against ROT-induced cells.

Cell viability assay

C6 cell viability was determined by MTT quantitative assay according to Van Meerlo *et al*. [40]. C6 cells were incubated with ROT (20 µM) and the simultaneous treatment with SES at different concentrations (10-100 µM) were carried out and incubated. After 24 hours, 10 µl of MTT was added to each well and incubated for 4 hours at 37°C. The absorbance was read at 570 nm. The percentage of cell viability was calculated by the formula:

$$\text{Cell viability (\%)} = \left[\frac{\textit{Optical Density of Test (570 nm)}}{\textit{Optical Density of Control (570 nm)}}\right] \times 100$$

Determination of intracellular reactive oxygen species (ROS)

The intracellular ROS generation induced by ROT in C6 cells was determined using DCFH-DA according to the method described by Wang and Joseph [41]. C6 cells were plated at a density of $1x10^5$ cells/well. The cells were exposed to ROT (20 µM) with and without SES (50 µM) at 37°C. After 24 hours, the media was removed and phosphate-buffered saline (PBS), pH 7.2 was used to wash the cells. The cells were treated with 10 µM of DCFH-DA and incubated for 1 hr at 37°C. The fluorescence was observed under a fluorescent microscope and measured at excitation and emission wavelength of 485 and 530 nm.

Changes in mitochondrial membrane potential

The recorded change in the mitochondrial membrane potential in C6 cells was visualized using rhodamine 123 according to the method described by Baracca *et al*. [42]. C6 cells were plated at a density of $1x10^5$ cells/well and exposed to ROT (20 µM) with and without SES (50 µM). The cells were washed with PBS

after 24 hrs. Then the cells were incubated with 5µg/ml of rhodamine 123 at 37°C for 30 mins. The fluorescence was observed under a fluorescent microscope and measured at excitation and emission wavelength of 488 and 525 nm.

Nuclear morphology assessment

The alterations in the nuclear morphology of C6 cells were observed by DAPI staining method of Morikawa and Yanagida [43]. C6 cells were plated at a density of $1x10^5$ cells/well. The cells were exposed to ROT (20 µM) with and without SES (50 µM) and incubated at 37°C. After 24 hrs, the media was removed and PBS was used to wash the cells. The cells were treated with 1µg/ml DAPI fluorescence dye and incubated for 1 hr at 37°C. The fluorescence was observed under a fluorescent microscope and measured at excitation and emission wavelength of 350 and 470 nm.

Animals

Male Wistar albino rats (150-180 g) were employed in the study. Rats were maintained at a temperature of 24±2ºC, in a 12 h dark/12 h light cycle, with food and water *ad libitum*. The studies were carried out with the guidelines given by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi (India). The Institution Animal Ethical Committee of Sathyabama University, Chennai approved the protocol of the study (SU/CLATR/IAEC/VI/034/2016).

Experimental design

The rats were divided into 5 groups, each containing 6 rats.
Group I: Vehicle (DMSO in corn oil intraperitoneal + Saline intraperitoneal) for 60 days.
Group II: Rotenone (3 mg/kg.B.wt intraperitoneal) for 60 days.
Group III: Co-treatment Rotenone (3 mg/kg.B.wt intraperitoneal) + Sesamol (50 mg/kg.B.wt intraperitoneal) for 60 days.
Group IV: Co-treatment Rotenone (3 mg/kg.B.wt intraperitoneal) + Sesamol (50 mg/kg.B.wt intraperitoneal) + L-DOPA (10 mg/kg.B.wt oral) for 60 days.
Group V: Co-treatment Rotenone (3 mg/kg.B.wt intraperitoneal) + L-DOPA (10 mg/kg.B.wt oral) for 60 days.

Histopathological analysis

The mid brain samples obtained from the experimental rats were fixed in 10% formalin for 3-6 hours. The tissue sections were embedded in paraffin wax and sectioned at 5-6 µm thickness and the sections were stained with Hematoxylin-Eosin solution [44] for microscopic observation.

Statistical analysis

The statistical analysis was performed using SPSS version 20 from IBM. The results were expressed as mean ± SD. One-way analysis of variance was applied to the data and the significance of the results was derived by running post hoc test. The $p<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Astrocytes are involved in the several functions of central nervous system and play an effective role in neurodegenerative diseases [45]. In general, neurons cannot survive without close interaction with astrocytes. During activation, the reactive glial cells produce a number of potentially toxic substances [46] which are harmful to neurons. In this study, we evaluated the protective effect of SES on ROT-induced C6 cells and rat brain. The cell viability was decreased with ROT (20 µM) treatment. The maximum viability of C6 cells was found at 50 µM SES when co-treated with ROT and on further increase of SES concentration at 100 µM, the viability was at steady state. Figure 1 and 2 shows the effect of SES on viability of C6 cells. SES (100 µM) alone treated C6 cells show similar results as vehicle control. Giuliani et al [47] reported that guanosine, a natural compound acting at the glial level could be a promising agent against neurodegeneration.

The correlation between ROT-induced ROS generation and mitochondrial impairment was observed by Radad et al [48] in primary dopaminergic culture. C6 cells induced with ROT leads to ROS production as shown in the experiments with the fluorescent dye DCFH-DA (Figure 3). C6 cells exposed to ROT shows a significant increase in DCF fluorescence whereas there is decreased fluorescence in cells co-treated with ROT (20 µM) and SES (50 µM). Co-treatment with SES shows a significant loss in green fluorescence which suggests that ROS generation was inhibited. SES (100 µM) alone treated C6 cells show similar results as vehicle control. Stief [49] reported that in biological systems, ROS can be formed by various factors like inflammation, pro-oxidative enzyme systems, lipid peroxidation, pollutants, etc. Accumulating evidences also indicates that astrocytes are also vulnerable to ROS [50-52]. This depicts that astrocytes damage may be a more potential pathological event causing degeneration of neighbour dopaminergic neurons.

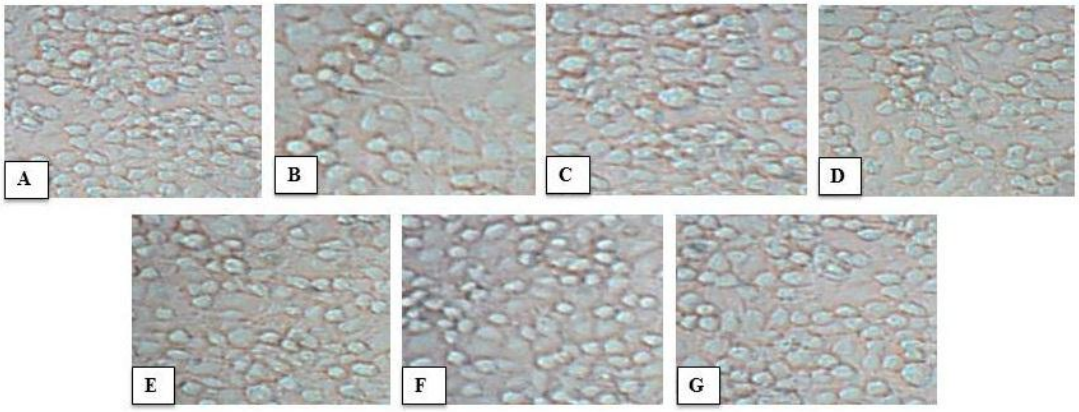
Rhodamine 123 staining indicates the mitochondrial membrane potential in C6 cells (Figure 4). ROT has decreased the mitochondrial membrane potential in C6 cells. Reichert et al [53] have also studied that ROS can cause astrocyte mitochondrial membrane potential depolarization and mitochondrial membrane potential transition. Decreased fluorescent intensity represented a drop in the mitochondrial membrane potential, when the C6 cells were induced with ROT (20 µM). The co-treatment with SES (50 µM) significantly inhibited the decrease in mitochondrial membrane potential caused by ROT. SES (100 µM) alone treated C6 cells show similar results as vehicle control. Kubik and Philbert [54] demonstrated that disturbances to astrocytic mitochondria can negatively create an impact on neuronal function.

DAPI staining indicates the nuclear morphology of C6 cells (Figure 5). On ROT induction, the nuclear damage was found to be high in C6 cells. Gupta et al [55] has also observed that 6-hydroxydopamine can cause chromatin condensation and DNA damage in C6 cells. Decreased nuclear damage was found during the co-treatment with SES (50 µM). SES (100 µM) alone treated C6 cells show no alterations in nuclear morphology and are similar to vehicle control.

Hematoxylin and eosin staining (Figure 6) indicates the histopathology of mid brain of vehicle treated and experimental group of rats. Dopamine releasing mid brain neurons are associated with the fundamental and complex brain functions [56]. Mid brain section of vehicle treated rats (Group I) reveals the normal architecture whereas ROT-induced rats (Group II) shows pathological changes like cellular inflammation, degeneration of cells and cytoplasmic vacuolation which coincides with the results of Reenu Jacob et al [57]. SES treated rats (Group III) shows the recovery from cellular inflammation, degeneration and vacuolation. SES + L-DOPA treated rats (Group IV) shows slight reduction in degeneration and vacuolation. L-DOPA treated rats (Group V) shows similar pathological changes as Group II rats. Neha Sharma and Bimla Nehru [58] observed that vitamin E, an efficient free radical scavenger has reversed the ROT-induced histopathological changes. Jolanta Dorszewska et al [59] observed that long-time therapy of L-DOPA, can cause side effects in the form of increased toxicity and inflammatory response.

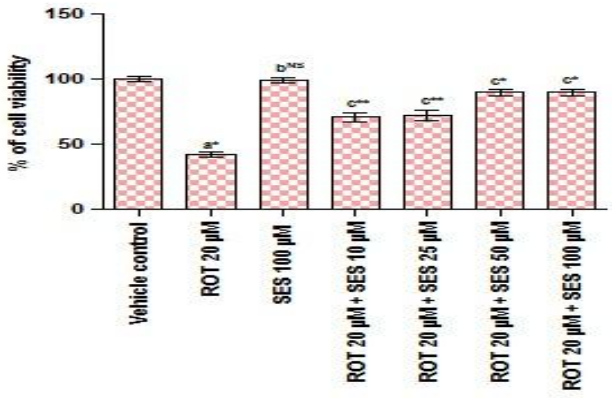
Thus, ROT has caused cell death, ROS generation, mitochondrial impairment, nuclear damage in C6 cells and brain damage in rats. SES has reduced the ROT-induced damage in this study, indicating its neuroprotective effect on PD pathology.

Fig 1. Shows the result of cell viability assay in C6 cells.



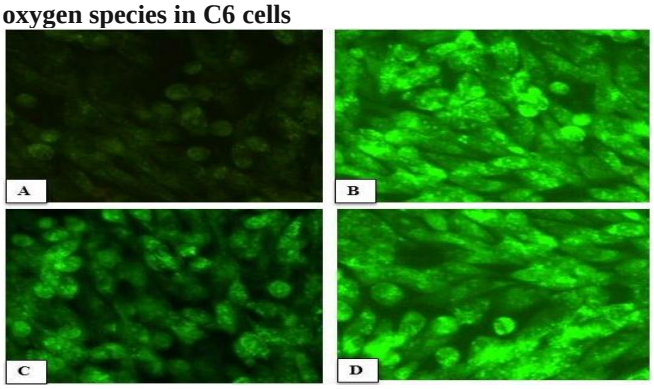
A- Vehicle control, B- Rotenone 20 µM shows increased cell death as compared to vehicle control cells, C- Sesamol 100 µM, D- Rotenone 20 µM + Sesamol 10 µM, E- Rotenone 20 µM + Sesamol 25 µM, F- Rotenone 20 µM + Sesamol 50 µM, G- Rotenone 20 µM + Sesamol 100 µM. (D,E,F,G)- shows decreased cell death as compared to Rotenone 20 µM induced cells. Magnification- 40X.

Fig 2. Shows the percentage of cell viability. Statistical significance: *P<0.001, **P<0.01 and ***P<0.05; NS-non-significant; n=3



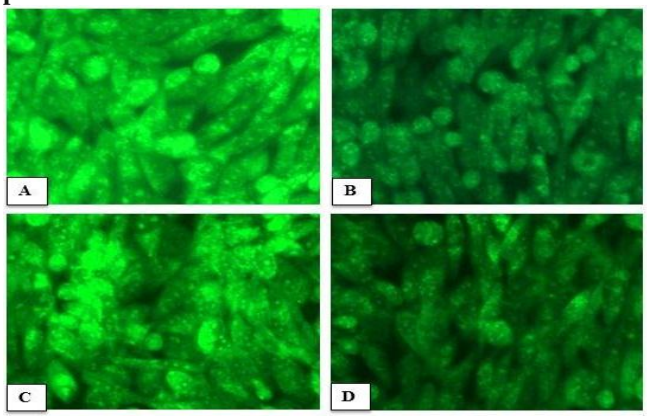
a- comparison between Vehicle control and Rotenone
b- comparison between Vehicle control and Sesamol
c- comparison between Rotenone and Rotenone + Sesamol.

Fig 3. Shows the determination of intracellular reactive oxygen species in C6 cells



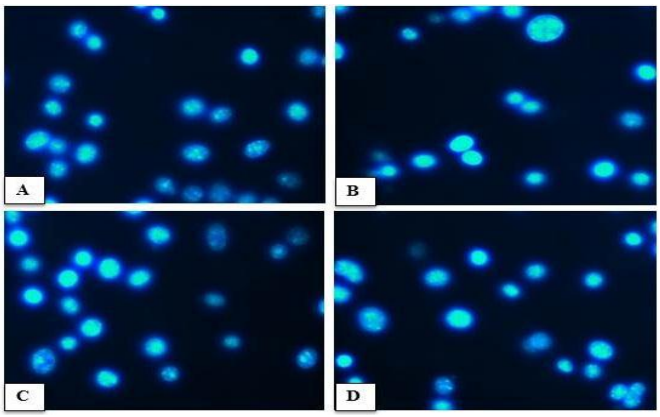
A- Vehicle control, B- Rotenone 20 µM shows increased level of reactive oxygen species as compared to vehicle control cells, C- Sesamol 100 µM, D- Rotenone 20 µM + Sesamol 50 µM shows decreased level of reactive oxygen species as compared to Rotenone 20 µM induced cells. Magnification- 40X

Fig 4. Shows the changes in mitochondrial membrane potential in C6 cells.



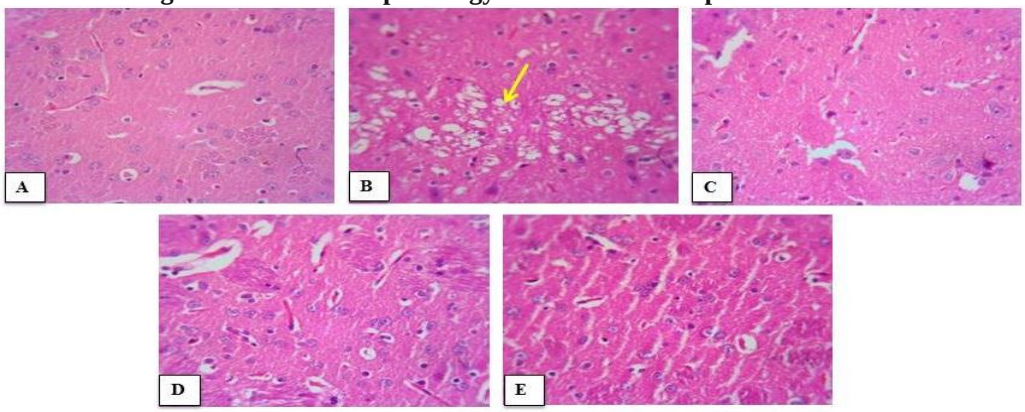
A- Vehicle control, B- Rotenone 20 µM shows decreased mitochondrial membrane potential as compared to vehicle control cells, C- Sesamol 100 µM, D- Rotenone 20 µM + Sesamol 50 µM shows increased mitochondrial membrane potential as compared to Rotenone 20 µM induced cells. Magnification- 40X

Fig 5. Shows the nuclear morphology assessment in C6 cells.



A- Vehicle control, B- Rotenone 20 µM shows increased nuclear damage as compared to vehicle control cells, C- Sesamol 100 µM, D- Rotenone 20 µM + Sesamol 50 µM shows decreased nuclear damage as compared to Rotenone 20 µM induced cells. Magnification- 40X

Fig 6. Shows the histopathology of mid brain of experimental rats.



A- Group I: Vehicle-treated rats reveals the normal architecture, Group II: Rotenone-induced rats (3 mg/kg.B.wt) shows pathological changes like cellular inflammation, degeneration of cells and cytoplasmic vacuolation (indicated by arrow), Group III: Rotenone (3 mg/kg.B.wt)+ Sesamol (50 mg/kg.B.wt) shows the recovery from cellular inflammation, degeneration and vacuolation, Group IV: Rotenone (3 mg/kg.B.wt)+ Sesamol (50 mg/kg.B.wt)+ L-DOPA (10 mg/kg.B.wt) shows slight reduction in degeneration and vacuolation, Group V: Rotenone (3 mg/kg.B.wt) + L-DOPA (10 mg/kg.B.wt) shows similar pathological changes as Group II rats.Magnification- 40X

CONCLUSION

Our results show that SES is an efficient antioxidant with therapeutic potential has prevented C6 cells and brain from damage. Thus, the emerging roles of astrocytes in the pathogenesis of PD establish a stimulating improvement in encouraging novel therapeutic goals.

ACKNOWLEDGEMENT

Senior Research Fellowship by the Department of Science and Technology- INSPIRE, New Delhi, India to Ms.D.Rohini is gratefully acknowledged.

ABBREVIATION

PD, Parkinson's disesase; ROT, Rotenone; L-DOPA, (L-dihydroxy phenylalanine); SES, Sesamol; MTT, 3-(4,5-dimethylthiazol-2- yl) - 2, 5 - diphenyl - tetrazolium bromide; DMSO, Dimethyl sulphoxide; DMEM, Dulbecco's modified Eagle's medium; FBS, Fetal bovine serum; NCCS, National Centre for Cell Science; ROS, Reactive oxygen species; DCFH-DA, dichlorofluorescein diacetate; PBS, Phosphate-buffered saline; DAPI, 4',6-diamidino-2- phenylindole; ATP, adenosine triphosphate; GFAP, glial fibrillary acidic protein; RNS, Reactive nitrogen species; CPCSEA, Committee for the Purpose of Control and Supervision of Experiments on Animals.

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