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Resveratrol with an adjunct for improved maintenance of mitochondrial homeostasis and dopamine neuronal rescue in neurodegeneration.

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Abstract

Background: Neurodegeneration, a key aspect of Parkinson's disease, is a progressive neurological disorder associated with the loss of dopamine-generating brain cells that results in a complex array of symptoms; bradykinesia, resting tremor, rigidity and postural instability. Its main hallmark is progressive loss of motor control. Mitochondrial dysfunction and the dysregulation or over activation of enzyme protein kinase CDK5 account for neurodegeneration to a large extent. Currently available therapies are of reducing symptoms only and do not address neurodegeneration. Resveratrol, a multi target potency antioxidant along with an adjunct, could be a potential therapy to treat neurodegeneration possibly by maintaining mitochondrial homeostasis and correcting CDK5 regulation.

Objective: To study the efficacy of a combination of resveratrol and an adjunct for the treatment of neurodegeneration in rotenone induced SH-SY5Y cell line models of Parkinson's disease.

Materials and Methods: The improved cell viability with drug combination was determined by Sulforhodamine b assay. The effect of drug combination to attenuate the oxidative stress was determined by DCFDA staining and quantification. Intracellular antioxidant enzymes like glutathione peroxidase and super oxide dismutase were determined using spectrophotometer. CDK5quantification was done by ELISA analysis

Results: The improved efficacy of the combination of resveratrol with an adjunct against mitochondrial dysfunction and CDK5 dysregulation in neurodegeneration was found to be superior compared to individual drug treatments.

Key Words: CDK5, Drug combination, Mitochondrial dysfunction, Parkinson's disease, Resveratrol, ROS.

INTRODUCTION

Neurodegenerative diseases are group of complex disorders of the nervous system characterized by gradual and irreversible loss of brain functions. These disorders can affect the life of patients, their families and friends causing physical, emotional and social burden [1]. Parkinson's disease (PD) is one of the most common neurodegenerative diseases after Alzheimer's disease (AD). It causes progressive motor disability and cognitive dysfunction [2]. It is caused due to the loss of dopamine (DA) neurons in the substantia nigra (SN), as a result of, either due to mitochondrial dysfunction or due to abnormal accumulation of cytoplasmic alpha-synuclein (α-synuclein) protein inclusions called lewy bodies in the neurons [3, 4]. The major symptoms of PD include bradykinesia, rigidity and tremor along with additional motor and non motor characteristics [5]. Current prevalence rate of PD in the world is estimated to be 1-2 per 1000 of the population, which tends to increase with the increase in age [5].

In addition, multiple gene defects have also been described for both familial and age-related forms of PD, most of these converge on pathways that regulate mitochondrial homeostasis. These pathological conditions implicate mitochondrial dysfunction in disease progression [6]. In PD postmortem brain tissues, α-synuclein accumulation and increase in oxidative stress and disturbance in mitochondrial function was observed [7]. Moreover, dysfunction of antioxidant defense system due to depletion of antioxidant enzymes like glutathione peroxidase (GPX), superoxide dismutase (SOD) cause the fragmentation of mitochondria, which will eventually lead to the decline in respiration and mitochondrial complex 1 activity, ultimately causing neuronal death [8]. Additionally, DA neurons within the SN are particularly sensitive to oxidative damage from mitochondrial-derived reactive oxygen species (ROS) [9]. Therefore, mitochondrial quality control through proper balance of mitochondrial homeostasis proves to be vital for treating neurodegeneration in PD.

Cyclin dependent kinase-5 (CDK5) is a protein kinase enzyme encoded by the CDK5gene. CDK5 is associated with neuronal maturation and migration process and required for the proper development of the brain and is highly expressed in brain neurons and shown to be activated in neurodegeneration [10]. CDK5 over activation and dysregulation has been found to be responsible for oxidative stress which shows an important link between oxidative stress and CDK5 [11]. Further, CDK5 is found to be phosphorylated by c-Abl in oxidative stress which shows an important link between active c-Abl, oxidative stress and CDK5 [12]. Hence, regulation of CDK5 and its phosphorylation could be considered as a viable therapeutic option for PD.

The pharmacological treatments of PD currently available are all of symptomatic therapy [13]. The major drugs available for the treatment of PD motor symptoms include levodopa (L-DOPA), dopamine agonists, monoamine oxidase (MAO) B inhibitors, anticholinergic agents,

amantadine and catechol-O-methyl transferase (COMT) inhibitors. In practice, these drugs are only beneficial to reduce the symptoms by working on either maintaining the balance between dopamine and acetyl choline or through dopamine replenishment. This does not appear to address the issue of neurodegeneration in PD [14, 15] that leads to further severe neurodegeneration which ultimately results in serious decline of motor activity and cognition. Therefore, developing a treatment strategy that can stop or slow the process of neurodegeneration holds a great potential in PD.

Resveratrol is a polyphenolic compound with antioxidant, anti-inflammatory, anti-apoptotic and neurotrophic properties [16], which could produce neuroprotective effects against neurodegeneration by controlling the oxidative and inflammatory mechanisms in the pathogenesis of PD [17].Maintenance of mitochondrial quality through the proper balance of biogenesis and degradation within the SN, as well as other brain regions is a pivotal step in preventing neurodegeneration in both familial and age-related PD [18]. Tyrosine kinase (c-Abl) inhibitor reduces c-Abl mediated phosphorylation of CDK5, and could be considered as a potent adjunct to resveratrol for the treatment of neurodegeneration by inhibiting the activity of the c-Abl/CDK5/dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP-32) signaling pathway in the striatum [19].

Hence, PD is a neurodegenerative disease that occurs due to multiple etiologies. Combination of drugs that target different molecular etiologies of PD could offer better efficacy compared to single drug therapy, to stop or halt the process of neurodegeneration [20]. In the current study, we therefore, have studied the potency of resveratrol (natural antioxidant) along with an adjunct (a tyrosine kinase inhibitor) for treating neurodegeneration in PD, with suitable cell line.

MATERIALS AND METHODS

Cell culture

The SHSY-5Y cell line is an excellent model of dopaminergic cells, found to express tyrosine hydroxylase, dopamine β-hydroxylase and dopamine transporter [21]. SH-SY5Y (neuroblastoma) cells (ATCC Cat# CRL-2266, RRID:CVCL_U924) were procured from National Centre for Cell Sciences (NCCS), Pune, India, and maintained in Dulbecco's modified Eagles medium (DMEM) (Gibco, Invitrogen).The cell lines were cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing, Penicillin (100U/ml), Streptomycin (100µg/ml) and Amphotericin- B (2.5µg/ml). Cultured cell lines were kept at 37ºc in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany).

Antibody Reporting

Antibody Characterization

CDK5 Polyclonal Antibody detects endogenous levels of CDK5 protein, using ELISA.

Determination of cell viability by Sulforhodamine b (SRB) assay

Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, 100µl cell suspension ($5x10^4$ cells/well) was seeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO_2 incubator. 1mg of sample was weighed and dissolved in 1mL DMEM using a cyclomixer. The sample solution was filtered through 0.22 µm millipore syringe filter to ensure the sterility. After 24 hrs the growth medium was removed, the cells were treated with rotenone (50nM) and incubated for 1 hr. The induced cells were then exposed with samples, dasatinib- 0.25µM, resveratrol-1.25µM, dasatinib + resveratrol, in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO_2 incubator. Untreated control cells and rotenone induced (50nM) cells were also maintained. Entire plate was observed after 24 hrs of treatment in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observation was recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

In the well plate without removing the cell culture supernatant, 100µl of cold 10% trichloroacetic acid (TCA) was added and incubated at 4ºC for 1 hr. After which, the plates were washed four times with slow running tap water and any excess water was removed using paper towels. The plate was dried using a blow dryer to completely dry them. Once the plates were dried, 100µl of 0.057% SRB solution was added to each well. The stain was allowed to be incubated for 30 min followed by brief rinsing the plate four times with 1% acetic acid to remove any excess unbound dye. This was followed by addition of 200µl of 10mM Tris base solution (pH 10.5) to each well. The plates where then placed in a shaker for 5 min to solubilize the protein-bound dye. The optical density (OD) was read in a microplate reader at 510nm [22].

The percentage of growth inhibition was calculated using the equation:

Percentage of viability= Mean Optical density (OD) of samples × 100/ Mean OD of control.

Table 1

S.No	Name of Antibody	Immunogen	Manufacturer, catalogue, species, monoclonal or polyclonal	Concentration of antibody used
1	CDK5antibody	Synthesized peptide derived from CDK5. at AA range:1-80	Immunotag,ITT0835, rabbit, polyclonal	1/5000 in ELISA

Treatment groups

The different treatment groups selected for the study were Group I: control; Group II: rotenone 50nM; Group III: resveratrol 1.25µM Group IV: dasatinib 0.25µM; Group V: combination of resveratrol 1.25µM + dasatinib 0.25µM. These groups were maintained for all the experiments in the present study.

Estimation of oxidative stress -ROS measurement (DCFDA)

The cells were trypsinized (500µl of 0.025% Trypsin in phosphate buffer solution/Ethylenediaminetetraacetic acid (PBS/ EDTA) solution) for 2 min and transferred to T flasks in complete aseptic conditions. Samples were added and incubated for 24 hrs. The cells were washed with PBS and added with 50 µl of 2',7'-Dichlorodihydrofluorescein diacetate (DCFDA) and incubated for 30 min. Excess dye was washed with PBS and the fluorescence was observed in blue filter of a fluorescent microscope (Olympus CKX 41 with optika pro 5 camera) [23].

Estimation of GPX

The extent of amelioration of oxidative stress can be estimated by comparing the levels of antioxidant enzyme, GPX, using spectrophotometry [24]. GPX catalyzes the oxidation of reduced GSH to its oxidized form which reacts with nicotinamide adenine dinucleotide phosphate (NADPH) and gets converted to the oxidized form of nicotinamide adenine dinucleotide phosphate (NADP) and two molecules of reduced glutathione which is measured spectrophotometrically at 340nm. Cell lysate (50µl) was mixed with 3ml of the reaction mixture [1mg of β -NADPH +1mM glutathione reduced]. It was then mixed by inversion, equilibrated to 25^0C and the absorbance was monitored at 340nm until constant mixing. The tube containing 3ml of the reaction mixture and 50µl of phosphate buffer with dithiothreitol at pH 7 was taken as blank. Hydrogen peroxide (H_2O_2) (50µl of 0.042 %) was added to these tubes. Decrease in the absorbance was measured after 5 min in a spectrophotometer (Cary 60, agilent) and the enzyme activity was calculated using the equation:

Enzyme units= (▲ A340nm/min Test - ▲ A340nm/min Blank) X 2 X 3.1 X df /6.22×0.05

Where, 2 = 2µmoles of GSH produced per µmole of β NADPH at 340nm; 3.1= Total volume (in ml) of assay; df = dilution factor; 6.22 = millimolar extinction coefficient of β NADPH at 340nm; 0.05 = volume (in ml) of enzyme used.

Estimation of SOD

The enzyme SOD catalyzes the dismutation of superoxide into oxygen and H_2O_2. As such, it is an important antioxidant defense in nearly all cells exposed to oxygen. 50µl of cell lysate were added to the reaction mixture containing 50mM phosphate buffer (7.8), 45µM methionine, 5.3 mM riboflavin and 84µM potassium ferric cyanide. The tubes were then incubated at 25^0C for 10 min and the absorbance was read on spectrophotometer at 600nm.

Percentage of Inhibition = ((Control-Test)/Control) X 100

Enzyme Units = Percentage Inhibition/ 50

Estimation of CDK5

Indirect ELISA was used to measure the total CDK5 concentration in treated cells. Cell free supernatants from all the groups were collected in a 96 well plate and it was coated and 200µl of blocking buffer of composition 0.2% gelatin in 0.05% Tween 20 (merck; Germany) in PBS (freshly prepared) was also added (1h at room temperature). Then it was washed with PBS TWEEN (2 times) and added 100µl of the primary antibody (CDK5) (ImmunoTag St.Louis, MO, USA, Cat# ITT0835) and left for 2 hrs at room temperature. Primary antibody was then collected and again washed with PBS TWEEN (2 times). Next, the secondary antibody (HRP Conjugate, Santacruz,USA) of 100µl was added and left for 1h at room temperature. It was then washed twice with PBS TWEEN. O- dianizdine hydrochloride (Sigma Aldrich,USA) (200µl) was then added and left for 30 min at room temperature and added blocking buffer of composition 1mg/100ml methanol + 21ml citrate buffer pH 5 + 60ml H_2O_2. By adding 5N HCl (50µl), the reaction was stopped and then the optical density was read at 415 nm in an ELISA reader and expressed in terms of protein units.

Activity of Antibody = OD Value / Protein Concentration

Statistical Analysis

Statistical analysis was performed using Graph Pad Prism 6 (GraphPad Software, La Jolla, CA) (GraphPad Prism, RRID: SCR_002798). All data were expressed as the mean±SD. All experiments were performed with a minimum of three replicates. One way analysis of variance (ANOVA), with a Tukey post hoc analysis was used for statistical comparisons between treatment groups. Values of $P<0.05$ were accepted to be statistically significant.

RESULTS

Fixing of dose based on cell viability by Sulforhodamine b assay

A sub lethal dose of 50 ηM rotenone was selected in accordance with previous findings which retained a cell viability of 58.59%. The optimal dose of drugs selected show increase in cell viability as resveratrol 1.25 µM (68.33%), dasatinib 0.25µM (73.83%) and the combination of resveratrol 1.25 µM + dasatinib 0.25µM (71.56%). These doses were used for performing further experiments (Figure 1).

Effect of resveratrol with adjunct therapy on ROS fluorescence intensity

The rotenone (50ηM) induced group shows increase in ROS fluorescence intensity compared to control group. In the resveratrol treatment group, a decrease in the ROS fluorescence intensity was obtained. In the combination treatment, however, a better reduction in the ROS fluorescence intensity was obtained when compared to rotenone induced group. The drug combination was thus

found to exhibit better results in terms of reduction in ROS fluorescence intensity (Figure2).

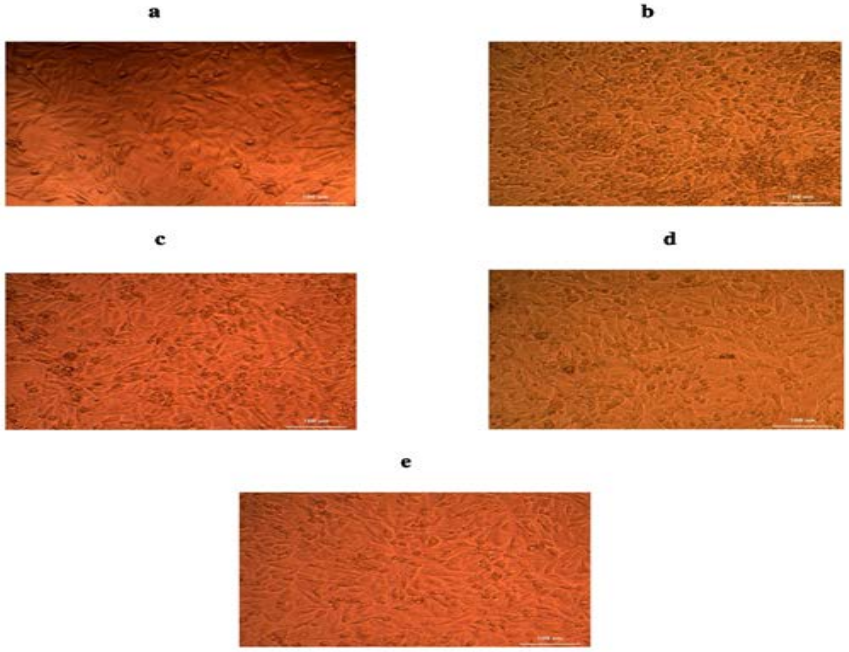

Figure 1 Percentage cell viability of different treatment groups (a. untreated control group; b. rotenone; c. rotenone + resveratrol; d. rotenone + dasatinib; e. rotenone + resveratrol + dasatinib).

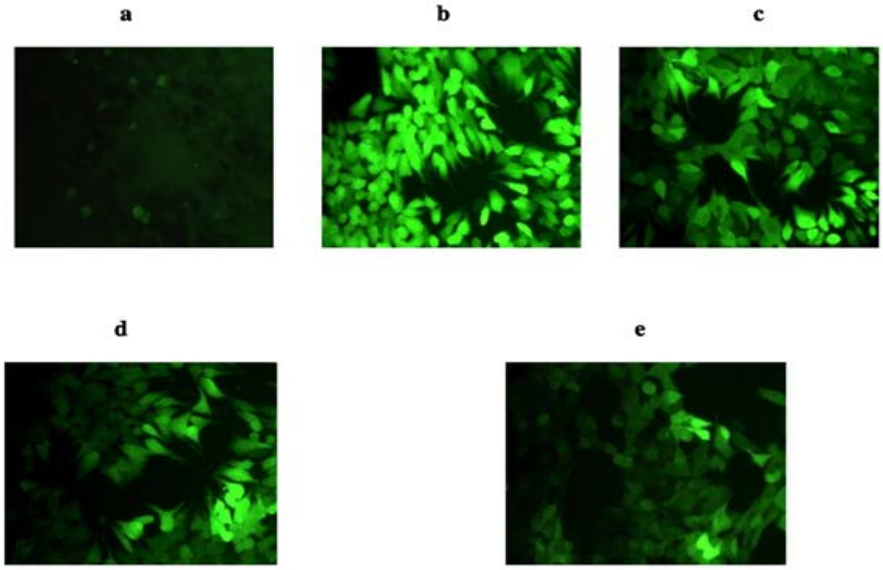

Figure 2 ROS fluorescence intensity of different treatment groups (a. untreated control group; b. rotenone; c. rotenone + resveratrol; d. rotenone + dasatinib; e. rotenone + resveratrol + dasatinib).

Effect of resveratrol with adjunct therapy on the antioxidant enzyme GPX

Rotenone (50ηM) induced group exhibited a significant (P<0.001) decrease in GPX levels (0.001716 ± 6.86) when compared with control group (0.09994 ± 0.0041). Resveratrol treatment with rotenone exposed SH-SY5Y cells, the GPX level was 0.02485± 0.00080 units/mg protein, whereas with adjunct dasatinib treatment, the GPX level was.006707 ± 0.0014 units/mg protein. Drug combination group, however, showed a significant increase in GPX level (0.02799± .0009) when compared with the rotenone induced group (P< 0.001). The combination therapy thus exhibits better results in GPX enzyme level (Figure 3).

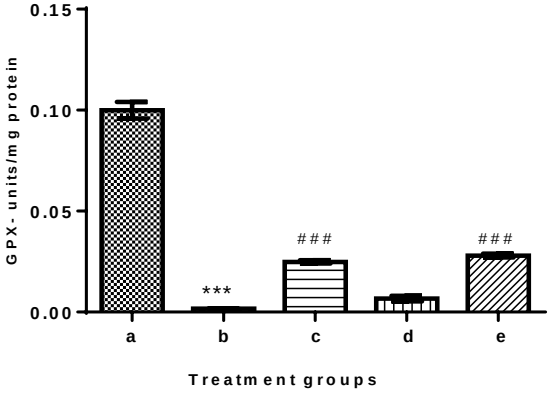

Figure 3 GPX intensity in different treatment groups (a. untreated control group; b. rotenone; c. rotenone + resveratrol; d. rotenone + dasatinib; e. rotenone + resveratrol +dasatinib). The error bars represent mean ± SD value from three independent experiments (n=3) (***P<0.001, compared with control, ###P<0.001 compared with rotenone).

Effect of resveratrol with adjunct therapy on the antioxidant enzyme SOD

Rotenone (50ηM) induced group exhibited a significant (P<0.001) decrease in SOD levels (0.08334 ± 0.0031) when compared with control group (0.3673 ± 0.0027). Resveratrol treatment with rotenone exposed SH-SY5Y cells, the SOD level was 0.1061 ± 0.0028 units/mg protein, whereas with adjunct dasatinib treatment, the SOD level was 0.09955± 0.0058 units/mg protein. Drug combination group, however, showed a significant increase in SOD level (0.1074 ± 0.0034) when compared with the rotenone induced group (P< 0.001). The combination therapy thus exhibits better results in SOD enzyme level (Figure 4).

Effect of resveratrol with adjunct therapy on CDK5

Rotenone induced (50ηM) untreated group showed a significant (P<0.001) increment in CDK5level (0.8750 ± 0.0034) when compared with control group (0.2076 ± 0.0030). Resveratrol when treated with rotenone exposed SH-SY5Y cells, the CDK5level was 0.2776±0.0031 units/mg protein, whereas for adjunct dasatinib, it was 0.3147± 0.0030 units/mg protein. The combination group, however, showed a CDK5level of 0.2393± 0.0026 units/mg protein which is significant (P< 0.001) when compared to untreated rotenone induced group. The combination therapy was thus found to exhibit better results for CDK5regulation (Fig 5).

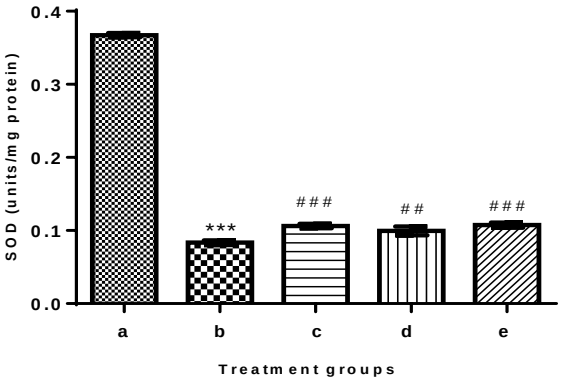

Figure 4 SOD intensity in different treatment groups (a. untreated control group; b. rotenone; c. rotenone + resveratrol; d. rotenone + dasatinib; e. rotenone + resveratrol + dasatinib). The error bars represent mean ± SD value from three independent experiments (n=3) (***P<0.001, compared with control, ###P<0.001, ##P<0.01compared with rotenone).

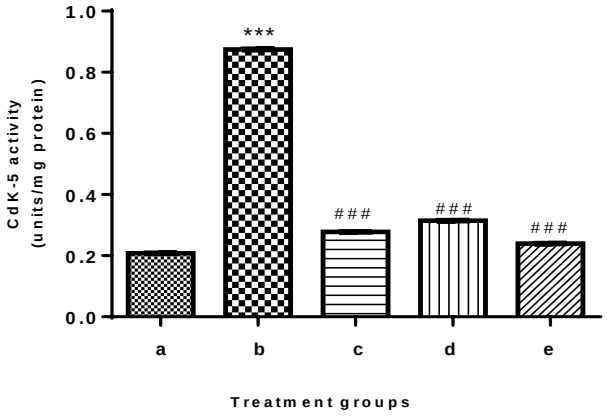

Fig 5 CDK5level measured in different treatment groups (a. control group; b. rotenone; c. rotenone + resveratrol; d. rotenone + dasatinib; e. rotenone + resveratrol + dasatinib), The error bars represent mean ± SD value from three independent experiments (n=3) (***P<0.001, compared with control, ###P<0.001, compared with rotenone).

DISCUSSION

Recent research has suggested that mitochondrial dysfunction, production of free radicals and increased oxidative stress are among the main causative factors responsible for dopamine cell death and neurodegeneration [25, 26]. Further, the elevation of CDK5expression and activation in the dopamine neurons of SN in neurodegeneration has been observed in various *in vitro* and *in vivo* studies [27, 28, 29]. Mitochondrial biogenesis and maintaining anti-oxidant defense system along with CDK5 regulation, therefore, hold the key as potential targets for the treatment of neurodegeneration in PD.

Mitochondrial homeostasis and production of ROS in cells are controlled by proper anti-oxidant defense system managed by enzymes like GPX, SOD etc. [30, 31]. The

present study reveals that the combination of resveratrol and adjunct is very potent in maintaining mitochondrial homeostasis by regulating ROS production and revamping the level of anti-oxidant enzymes like GPX, SOD in rotenone exposed cells, which is vital for the treatment of neurodegeneration.

In addition, the inhibition of CDK5 has been reported to reduce the loss of dopaminergic neurons in the SN and reduce functional impairment of the animals exposed to MPTP [28, 32]. In the present study, the resveratrol with adjunct drug combination shows favorable results in terms of regulating the CDK5level thereby regulating its phosphorylation in the rotenone exposed cells and, therefore, it proves to be a good candidate for the treatment of neurodegeneration.

CONCLUSION

Our study aims to target different pathways to halt or slow the neurodegeneration process in PD by acting on oxidative stress induced mitochondrial dysfunction and maintaining normal cellular process. It really differs from the current or existing treatment knowledge by trying to find a strategy to cure the disease condition than addressing only the symptoms. Consequently, we are using the available data for utilizing a strong natural antioxidant having distinctive targets alongside an adjunct, which is an endeavor to repurpose a current anticancer medication. We would hope that least effective dose combination of resveratrol and an adjunct, with the help of ideal formulation using convenient route could be an effective treatment strategy for neurodegeneration and for the betterment of ailing patients all over.

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