Evaluation of Daidzein Against Rotenone Induced Parkinson's Disease and a Potential Involvement of Mitochondrial Biogenesis.

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Abstract

Parkinson's disease (PD) ranks as second most prevalent neurodegenerative disorder but is devoid of neuroprotective treatment. Approaches with disease modifying ability with symptomatic relief has become an utmost necessity. Further multifactorial nature of PD presents challenges for efficacy evaluation of any potential test compound. The stated study makes an attempt to address these issues by employing a rotenone induced PD model involving a bilateral intranigral stereotactic rotenone injection for evaluation of the neuroprotective efficacy of Daidzein (DZ). DZ a soy isoflavone, is known for its various health benefits viz. immunomodulation, cardiovascular effects etc. In this study, animals after intranigral rotenone (12 µg) injection, were treated with DZ at a dose of 5, 10 and 20 mg/kg for 30 days. The neurobehavioural evaluation comprised of Rota-rod, Open field and Barnes maze test. The biochemical analysis constituting oxidative stress (Reduced glutathione, superoxide dismutase, catalase and lipid peroxidation), inflammation (TNF-α), mitochondrial alteration (complex I activity and biogenesis) was conducted on mid-brain tissue after 30 days of treatment. The SN and striatum was also subjected to immunohistochemical analysis (IHC) for TH positive neurons and Glial Fibrillary Acidic Protein. The analysis revealed significant improvement by daidzein in motor co-ordination and attenuation in cognitive deficits due to rotenone. The biochemical assessment exhibited significant decrement in oxidative stress as well as inflammation. DZ treatment also prevented complex I inhibition and promoted mitochondrial biogenesis eventually contributing to the neuroprotection apparent in IHC. Thus, the results strongly corroborate the neuroprotective potential of DZ against rotenone induced model of PD.

Introduction

Neurodegenerative disorders such as Parkinson's disease (PD) are one of the most debilitating and prevalent disorders affecting patients worldwide. According to the Global Burden of Diseases, Injuries, and Risk Factors Study (GBD)-2016 published in *The Lancet Neurology*, the neurological disorders are the second most likely cause of deaths globally and are estimated to be the leading cause of disability (Ray Dorsey et al. 2018). The burden of neurological disorders is forecasted to increase due to the higher ageing population and population growth (Lonneke M L de Lau and Monique M B Breteler 2006). PD in particular is a universal disorder with an incidence rate of 4.5 to 19 per 1,00,000 population/ year (Kasten et al. 2007). The crude prevalence estimates reveal there are 328 per 1,00,000 persons in the Parsi community in Mumbai, India (Radhakrishnan and Vinay 2018). Such figures make it imperative to search for options for better treatment, prevention and management of these disorders globally.

PD as first described by James Parkinson in 1817, is a chronic progressive neurodegenerative disorder (Przedborski and Dauer 2003). It is characterised by presence of predominant motor symptomatology usually in the form of tremors, bradykinesia, rigidity, postural instability. It also shows the presence of certain non-motor symptoms comprising hyposmia, rapid eye movements, sleep behaviour disorder, neuropsychiatric disturbances etc. Many of these symptoms are usually manifested before the motor symptoms and go unnoticed. The denervation of nigrostriatal tract and the consequential reduction in

striatal dopamine level due to progressive damage of dopaminergic neurons in the substantia nigra pars compacta (SNpc) forms the major pathophysiological trait of PD. This denervation leads to a compromised striato-pallidal and pallido-thalamic output pathway, which is responsible for the major motor deficits observed (M.S. Lima et al. 2012; Schapira et al. 2017).

PD is considered to be a multifactorial disorder. Oxidative stress, inflammation, ubiquitin-proteasome system defects, and mitochondrial dysfunction are implicated as the major contributors to PD pathogenesis. These effects collectively lead to cellular dysfunction ultimately resulting in neuronal degeneration (Betarbet et al. 2002). The mitochondrial dysfunction initiating from complex I inhibition and disruption of electron transport chain (ETC) forms the basis of PD pathogenesis (Franco et al. 2016; Grünewald et al. 2019). The available therapeutic treatment options for PD do not rectify the underlying cause of the disease thus rendering these treatments symptomatic. Therefore, currently the research focuses on the search for strategies which can slow or halt the disease progression as well as ease the symptoms and improve the quality of life of the patient. One such strategy is the inclusion of herbal phytoconstituents in the PD treatment strategy.

Daidzein (4',7-dihydroxy isoflavone) belongs to a family of isoflavone and it is one of the most widely consumed and studied type of phytoestrogen. Phytoestrogens with an isoflavone core have found to functionally mimic mammalian estrogens by binding to estrogen receptor (ER) such as ER β , ER α , and G Protein-Coupled Estrogen Receptor 1 (GPER1) (Adams et al. 2012; Morissette et al. 2018). It is most often found in nuts (peanuts), soybeans and soy-based products. Daidzein (DZ) is one of the major components of soy isoflavones which are known to have various health benefits such as immunomodulation and against diseases viz. cardiovascular disorders, osteoporosis, menopausal symptoms etc (Lo et al. 2007). Taking into consideration these favourable effects, DZ has been under investigation for its neuroprotective effect for various central nervous system disorders.

With respect to PD, DZ has been evaluated for its anti-inflammatory potential against the lipopolysaccharide (LPS) induced toxicity in BV-2 murine microglial cell line. The preventive effect conferred by DZ was also observed on dopaminergic N27 cell lines when exposed to conditioned media from BV-2 cells which were pre-treated either with daidzein or LPS. The results from both studies indicate that the neuroprotection bestowed was contributed by the ability of DZ to dampen the microglial activation process and inhibition of proinflammatory factors (Chinta et al. 2013). In another study performed by Lin et al. 2010, DZ inhibited 6-OHDA induced cellular toxicity and apoptosis in PC12 cells differentiated by nerve growth factor (NGF). They proved that the regulation of Caspases 8 and 3 contributed to the protective effect (Lin et al. 2010). Furthermore, the well documented neuroprotective effect of gonadal hormones through anti-inflammatory, antioxidant effects and epidemiological studies, specifically 17 β -estradiol, render DZ as a potential candidate in PD therapeutics (Morissette et al. 2018). Thus, our study focuses on the evaluation of DZ for its neuroprotective effect against rotenone induced Parkinsonian pathophysiology and symptoms, in conjunction with its mechanistic assessment.

Material And Methods

Daidzein (96%) was procured from AK Scientific, Inc. USA. Rotenone was obtained from TCI Chemicals Pvt. Ltd. (India). All other chemicals and reagents used in the experiments were of analytical grade. The tyrosine hydroxylase and glial fibrillary acidic protein primary monoclonal mouse antibody belonged to Abcam, (USA). The TNF- α elisa kit was procured from eBioscience, (USA). The reagents for qRT-PCR were procured from DNase, Invitrogen, iScript™ Select cDNA Synthesis Kit, Bio-Rad, SYBR® Green master mix, Bio-Rad.

Animals

The animal study protocol no. ICT/IAEC/2016/P02 was approved by the Institutional Animal Ethical Committee registered under the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), India. All the possible precautions were undertaken to minimize the number of animals included in the experiment as their suffering. 5-6 weeks old adult male Sprague Dawley (200-250 g) rats were procured from National Institute of Biosciences (NIBS), Pune-India. These were housed in animal house of Institute of Chemical Technology (ICT) under controlled laboratory conditions of 12 hour light-dark cycle with controlled temperature (25 $\pm$ 2°C) and relative humidity (50-70%) for at least one week prior to the stereotaxic surgery. All the animals were allowed *ad libitum* access to food and water.

Experimental design

For the present study, animals were randomly divided as per table 1

No.	Group	Treatment	No. of animals
1.	Group I	Vehicle control (DMSO), intranigral, bilateral	10
2.	Group II	Rotenone (12 μ g/ μ l), intranigral (bilateral)	10
3.	Group III	Sham control	10
4.	Group IV (DZ-5)	Rotenone (12 μ g/ μ l), intranigral (bilateral) + Daidzein (5 mg/kg) <i>p.o.</i>	10
5.	Group V (DZ-10)	Rotenone (12 μ g/ μ l), intranigral (bilateral) + Daidzein (10 mg/kg) <i>p.o.</i>	10
6.	Group VI (DZ-20)	Rotenone (12 μ g/ μ l), intranigral (bilateral) + Daidzein (20 mg/kg) <i>p.o.</i>	10
7.	Group VII (Standard control)	Rotenone (12 μ g/ μ l), intranigral (bilateral)+ Levodopa + Carbidopa (10 mg/kg) <i>p.o.</i>	10

Table 1: Animal grouping and distribution according to the respective treatment. *p.o.*- peroral

Stereotaxic surgery and drug treatment:

The animals were prepared for the surgery by induction of anaesthesia with 4% isoflurane, 0.5 L/min O₂ inhalational anaesthesia and maintained in it throughout the surgical procedure. Post anaesthesia, the hairs on the surgery area (scalp) were trimmed and the area was disinfected with povidone iodine. The animal was then laid on surgery board with straight head positioned. A midline sagittal incision was made on the scalp and the area below it was cleansed to expose the bregma. The position of Substantia nigra was pinpointed for drilling the burr holes according to the mentioned coordinates Anteroposterior (AP): -5.0 mm from the bregma; Mediolateral (ML): ± 2.1 mm from the midline; Dorsoventral (DV): 8.0 mm from the skull. 1 μ l Rotenone (12 μ g/ μ l) was infused bilaterally in SN (Paxinos and Watson 2004). The control group animals received DMSO. A sham control group received only burr holes in the skull. Post rotenone administration, the animals were kept on thermal pad, the cut on the scalp was sutured and covered with povidone iodine solution. Each animal was administered gentamicin (40 mg/kg, i.p.) and Tramadol (5mg/kg; i.m.) and returned to their cage for recovery. All animals received a recovery period of one week before the initiation of drug treatment. The treatment duration was of 30 days and each group received their respective doses per day as per the table no. 1. The animals were subjected to neurobehavioural analysis during the study duration which included Rotarod test, Open field test and Barnes Maze test as described in the Fig. 1.

Behavioural analysis

Rotarod test

The rota rod instrument (RR01 Plus, Orchid Scientifics, Nashik, India) was used to evaluate the motor impairment in the rat on 0th, 3rd, 15th and 30th day of the study (Carter et al. 2001). Briefly, the rat was placed on a rotating rod of the instrument at a fixed speed of 30 RPM. The time taken by the rat to fall down from the revolving rod was noted. A predetermined cut off time of 60 seconds (sec) was fixed for the study.

Open field Test

The open field is a square arena made up of plywood with a length and breadth of 72 cm and 36 cm height. Sixteen sub-square divisions of 18 $\times$ 18cm were plotted on the floor arena. The test was started by placing the test rat in the centre of the arena and its behaviour was observed for next 5 minutes. After observation the test rat was placed in its home cage and the apparatus was cleaned with 70% ethanol before placing the next test rat. The parameters observed on Day 0, 2 and 29 included number of line crossings (crossing the squares boundaries with both forepaws), number of rears (standing on its hind legs), grooming (rubbing the body with paws or mouth and rubbing the head with paws) and immobility time (Gould et al. 2009).

Barnes Maze Test

The Barnes maze consists of a circular platform with a diameter of 122 cm, consisting of 20 holes evenly punched on the perimeter. A box with its inside painted in black called "an escape box" was attached

under one of the holes below the platform. The rat was acclimatised initially by placing it in the centre of the maze into a start tube. After 30 sec, the rat was released from the start tube and the rat was allowed to enter the escape box within 3 min of the cut-off time where it was allowed to stay for 2 min. The first day (acclimatisation phase) was followed by 4 days of acquisition period. The rats were directed to the escape hole during these phases if they failed to locate its position within 3 min. The rats were provided with external cues for better learning. On the last day (probe trial), memory retention was evaluated without the escape box. The “escape latency time” i.e. the time (seconds) required for the rat to find and completely enter the escape box was noted during all the phases. The Barnes maze test was carried on Day 21 to 27 of the study period (Cohen et al. 2013; Li et al. 2013).

Midbrain processing and sample preparation

The rats were euthanized with CO₂ asphyxiation after 30 days of study. The midbrains of the rats from both hemispheres were dissected in ice cold conditions. The isolated mid-brains were rinsed in ice-cold isotonic saline for biochemical estimations and separated in to right and left regions. They were snap freezed with liquid nitrogen and stored in deep freezer till further investigations. For biochemical investigations, the mid-brain was homogenised in 10% w/v of ice-cold 0.1 M phosphate buffer saline (pH 7.4). It was centrifuged at 10,000 rpm at a temperature of 4°C for 15 min and used for estimations of biochemical parameters. For immunochemistry analysis, the rats were perfused with 4% paraformaldehyde and the whole brain was stored in it till further analysis.

Biochemical Analysis

Determination of oxidative stress by Superoxide Dismutase (SOD), Catalase (CAT) and Reduced Glutathione (GSH) analysis.

The measurement of SOD, Catalase and Reduced GSH was conducted to assess the oxidative stress in mid-brain due to rotenone administration. The activity of SOD was determined according to the method stated by (Nandi and Chatterjee 1988; Li 2012) with minor modifications. The inhibition of autooxidation of pyrogallol by SOD was measured by noting the rate of change in absorbance at 325 nm for 5 min and presented as unit activity/ mg protein. The Catalase activity was assessed by method described by (Sinha 1972; Aebi 1974) with minor changes. The decomposition of H₂O₂ was recorded in the terms of rate of decrease in the absorbance at 240 nm for 3 min and the results were expressed as unit activity/ mg protein. The quantification of reduced GSH was carried out according to the protocol described by (Sedlak and Lindsay 1968; Smith et al. 1988) with minor modification. The absorbance of the reaction mixture was read at 412 nm and the quantity was presented as μ mole/ mg protein.

Determination of Lipid Peroxidation (LPO)

The LPO in the mid-brain homogenate was determined by the spectrophotometric assessment of the Thiobarbituric acid reactive substances (TBARS) i.e. the LPO end product malondialdehyde (MDA) (Ohkawa et al. 1979; Reilly and Aust 1999). The absorbance of pink coloured organic layer from

the reaction mixture was determined at 532 nm and the LPO was expressed as nmol of MDA/mg of protein.

Determination of protein content

The protein concentration in the mid-brain homogenate was estimated using the Bradford's method with bovine serum albumin (BSA) standard curve. The colorimetric reaction was measured at 596 nm in a microplate spectrophotometer (Epoch, Biotek, USA) and expressed as mg/ml.

Immunohistochemistry analysis

The immunohistochemical analysis was performed on 4% paraformaldehyde-fixed, 5 µm-thick brain sections fixed on poly-L-lysine coated slide. The sections selected consists of SNpc and striatum. The fixed sections were subjected to through three changes of xylene for 30 min followed by rehydration with absolute alcohol. The endogenous peroxidase activity was quenched by incubating it with 3% hydrogen peroxide in methanol. The slides were further subjected to antigen recovery using citrate buffer at pH 6 for 15 min. After the initial steps the sections were processed separately for Tyrosine Hydroxylase (TH) positive neurons and Glial Fibrillary Acidic Protein (GFAP) detection.

For TH detection, the tissue sections were washed with Tris-buffered saline with Triton-X (TBST) (0.025% Triton X-100) and blocked with 10% normal saline and 1% BSA. The sections were further incubated overnight with primary monoclonal antibody for Tyrosine Hydroxylase (TH) at 4°C. The sections were washed with Tris buffer solution pH 7.4 after incubation and incubated with Poly- Horseradish peroxidase for 30 min. The brown colour change in the tissue was examined after its incubation with the substrate.

For GFAP detection, the antigen recovery was followed with a treatment with Phosphate buffered saline with 0.3 % Triton X-100 (PBST). The tissues were further incubated overnight with the primary monoclonal anti-GFAP antibody (ab10062) at 4°C. After the overnight incubation, the tissues were further incubated with secondary antibody Alexa fluor @ 488 (goat anti-mouse) for 1.5 hrs after a wash with PBS. The GFAP examination was carried out after being counterstained with DAPI (1.5% in PBS) after a wash with PBS (Fukuda et al. 1999; Sakuma et al. 2008).

The coded samples were evaluated for the intensity of staining and number of immune-positive cells under a light microscope at a magnification of 40 X for unbiased assessment.

Determination of TNF-α by ELISA

The TNF-α levels in midbrain was assessed as per the protocol guidelines provided in the ELISA kit by eBiosciences, USA. The TNF-α levels were expressed as pg/mg protein.

Mitochondrial complex I activity estimation

The protocol for mitochondrial complex I inhibition was utilized as described by (Spinazzi et al. 2012) with minor modifications. The mid brain homogenate prepared in sucrose homogenization was

used as a source of mitochondria. The activity of NADH: ubiquinone oxidoreductase was analysed by means of a spectrophotometric assay which estimates the complex I activity based on the rate of oxidation of NADH to its non-absorbing oxidized form NAD⁺ over the time of 4 minutes at 340 nm (the absorption maximum of NADH). The enzyme activity is calculated considering the extinction coefficient of NADH as 6.2 mM⁻¹ cm⁻¹.

Mitochondrial biogenesis

The mitochondrial biogenesis potential of the treatment was analysed by determination of the Mitochondrially Encoded Cytochrome C Oxidase I MtCO1/ COX1 target gene relative expression by RT-PCR. For assessment the total RNA from 50-100 mg mid-brain tissue was extracted using guanidinium-phenol-chloroform method. The extracted RNA purity and yield were determined by Nanodrop spectrophotometer (BioTek, USA). The RNA was further subjected to DNase I (Invitrogen, USA) treatment for 1 hr at 37°C and cDNA synthesis was carried out according to the oligo(dT) Bio-Rad kit protocol.

The qRT-PCR was performed in a Bio-Rad CFX96 Real-Time PCR Machine (Bio-Rad). The method employed Bio-Rad SYBR green master mix (iQSYBR, BioRad). The MtCO1/ COX1 relative expression was determined using gene-specific primer sequence of 5'- CCC CCG CTA TAA CCC AAT ATC AGA C-3' (forward); 5'- TGG GTG TCC GAA GAA TCA AAA TAG-3' (reverse) and normalized to 18s rRNA (Sigma) with a primer sequence of 5'-CAT TCG AAC GTC TGC CCT AT-3'(forward); 5'-GTT TCT CAG GCT CCC TCT CC-3' (reverse). The thermal cycling conditions were set as initial denaturation at 94°C for 3 min; for 35 cycles of 94°C for 30 secs, primer annealing at 60°C for 30 secs. The amplification reaction was run in triplicates with a no-template control and the DNA melt curve analysis was used to confirm product specificity. The relative gene expression was determined using the 2^{-ΔΔC_t} method for real-time PCR procedures and analysis following the MIQE guidelines (Livak and Schmittgen 2001; Bustin et al. 2009).

Statistical analysis

The results of the present study are represented as mean ± SEM. The data was statistically analysed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). The analysis was performed by applying one-way ANOVA followed by post-hoc Dunnett's Test. The Open field test and Barnes maze test were subjected to Two-way ANOVA followed by post hoc Bonferroni Test. The RT-PCR data was subjected to One-way ANOVA followed by post-ANOVA Tuckey's test. The significance level designated as per the calculations are ### p< 0.001 when compared with Group I i.e. vehicle control group, *, ** and *** when p<0.05, 0.01 and 0.001 respectively when compared with Group II i.e. Rotenone treatment group.

Results

Behavioural Analysis

Rota rod test

The baseline readings (Day 0) show relative equal rota rod activity (50-60 sec) by animals from each group as shown in Fig. 2. The readings on Day 3 after the initiation of treatment exhibit a decline in the rota rod performance by animals in all groups. This decline was observed to be significant ($P < 0.001$) and gradual over the entire study duration in the rotenone treated animals when compared to the vehicle control group. Whereas the DZ treatment at all the doses i.e. 5,10 and 20 mg/kg brought about a gradual improvement in the motor activity when examined on Day 15 and 30. This improvement was found to be significant ($P < 0.001$) when compared to the rotenone treatment group on the same days. The Levodopa + Carbidopa treatment as standard was also successful in significantly attenuating ($P < 0.001$) the motor impairment induced by rotenone.

Open field test

The exploratory behaviour, locomotion and general activity is analysed in Open field test by means of the four parameters viz. number of lines crossed in 5 min, immobility time, grooming and rearing exhibited by the animal. A significant impairment in the all the four parameters with rotenone treatment was observed over the entire duration of the study as observed in Fig. 3. This significance in depreciation was evident starting on day 2 after treatment initiation. The immobility in animals was gradually and significantly reduced by DZ at all the doses on Day 29 (DZ5: $P < 0.05$ and DZ10, 20: $P < 0.001$). Similar observations were also detected with respect to line crossing and rearing by animals. An effect on grooming activity in all animals was also noted on Day 2 of the treatment. This behaviour was found to be improved significantly only with DZ 20 treatment. The standard treatment overcame the impairment in these parameters significantly only on Day 29.

Barnes Maze Test

As mentioned previously BMT was employed to assess the effect of treatment on the cognitive function of the study animals and is illustrated in Fig. 4. The Day 3 of the BMT analysis displays a significant discrepancy ($P < 0.05$) in the escape latency between the rotenone and vehicle control group. The DZ treatment at dose 20 mg/kg showed a gradual improvement over the entire duration of the test similar to the control group. The difference was significant ($P < 0.05$) on the Day 5 of the study when compared to the rotenone treatment. The probe trial (Day 6) gives a measure of the memory retention of the location cues learned during the acquisition phase of BMT. As apparent from the results rotenone treatment group struggled to locate the box with a significantly high escape latency when compared to the vehicle control group ($P < 0.01$). The DZ treatment groups at all the doses displayed a significant reduction in the escape latency when compared to rotenone group, implying the ability of the animals to retain the memory ($P < 0.05$). The standard treatment group was ineffective in improving the cognitive function in animals as the difference in the escape latencies was not significant as compared to the rotenone treatment.

Biochemical Parameters

SOD, Catalase and Reduced GSH

The effect of rotenone on the cellular anti-oxidant defences and thus the induction of oxidative stress was measured with by analysing the activity of SOD, Catalase and Reduced GSH. Rotenone administration caused a significant reduction ($P<0.001$) in the activity of SOD, Catalase and reduced GSH when compared to the vehicle control group. This reduction was dose dependently and significantly prevented by DZ as observed in Fig. 5 a, b and c. The Levodopa + Carbidopa treatment was not able to ameliorate the reduction.

Lipid peroxidation

Lipid peroxidation assay measures the damage to cellular membranes due to oxidative stress in terms of the rise in the levels of metabolite Malondialdehyde (MDA). As apparent in the Fig. 5d, rotenone treatment led to a significant increase in the levels of MDA indicating the damage due to the oxidative stress ($P<0.001$). This damage was significantly restrained by DZ at all the doses. This preventive effect of Daidzein was better than levodopa and Carbidopa at the doses 10 and 20mg/kg ($P<0.001$).

Tumour necrosis factor- α

The TNF- α levels assessed in the midbrain provide a measure of the inflammatory response to the rotenone administration. As evident from the Fig. 5e, a significant rise in the levels have been observed due to rotenone when compared to the vehicle control group ($P<0.01$). Daidzein has found to negate this upsurge in TNF- α dose dependently with the significant decrease observed at the dose of 10 and 20 mg/kg ($P<0.01$ and $P<0.001$ respectively). The standard levodopa and carbidopa treatment were unable to prevent the increase in the TNF- α levels.

Mitochondrial complex I activity

The intranigral administration of rotenone lead to a significant decline in the complex I activity ($P<0.001$) in the midbrain of the rats as compared to the vehicle control group (Fig. 6a). The group DZ-10 ($P<0.005$) and DZ-20 ($P<0.01$) significantly restrained the inhibition of complex I activity due to rotenone treatment dose dependently. This prevention was not conferred by the standard levodopa and carbidopa treatment.

Mitochondrial biogenesis

The modulation in the COX1/ MtCO1 expression by the treatments was determined to assess the degree of mitochondrial biogenesis stimulated. As observed in the Fig. 6b, rotenone treatment brought about a downregulation in the gene expression of MtCO1 when compared to the vehicle control group ($P<0.005$). The daidzein treatment at all the doses upregulated the gene expression significantly ($P<0.001$) which was not observed with the standard treatment.

Immunohistochemistry analysis

Tyrosine hydroxylase immunoreactive neurons (TH-ir)

The TH immunohistochemistry of SN and striatum was performed to determine the degeneration of the dopaminergic neurons as illustrated in Fig 7 a and b respectively. The rotenone treatment elicited its toxicity as evident by the significant reduction (### $P < 0.001$) in the TH positive neurons in both SN and striatum. This neurodegeneration of nigrostriatal DA neurons was effectively prevented significantly and dose dependently by daidzein treatment. This prevention was better than that conferred by the levodopa and carbidopa ($P < 0.05$).

Glial Fibrillary Acidic Protein

GFAP is a classical astrocytic marker for detection of inflammation. The results of GFAP immunohistochemical analysis of the present study have been expressed in Fig 8. A significant elevation in GFAP levels ($P < 0.001$) was observed due to rotenone administration when compared to the vehicle control group. This elevation was significantly ($P < 0.001$) and dose dependently attenuated by daidzein which was better than the standard treatment of Levodopa and Carbidopa ($P < 0.005$).

Discussion

PD is an age related chronic progressive neurodegenerative disorder with an unmet need of a proper therapeutic management. PD remains to be incurable with an available treatment afflicted with side effects and devoid of long-term efficacy. These options, target the symptomatic management and do not address the underlying etiopathogenesis of PD. Therefore, the recent research focuses on developing newer and better strategies to overcome the shortcomings of current treatment along with disease modification (Oertel 2017; Charvin et al. 2018). The emphasis of our study was to appraise the protective potential of isoflavone Daidzein against neurodegeneration responsible for disorders such as Parkinson's Disease. To achieve this aim, we designed an experiment using rotenone as a Parkinsonism inducing agent. Rotenone belongs to isoflavanoid family and is the most toxic rotenoid member. A stereotaxic rotenone infusion in brain areas has been reported to induce dose dependent and a progressive degeneration in nigro-striatum which is TH specific and devoid of mortality. The acute intranigral rotenone administration is able to manifest a sustained neuropathological alteration in the nigro-striatal area akin to idiopathic PD (Saravanan et al. 2005; Xiong et al. 2009; Swarnkar et al. 2010). A bilateral infusion of 12 $\mu\text{g}/\mu\text{l}$ rotenone was found to result in depletion of about 60% of TH-ir neurons in SNpc manifesting pathological behavioural deficits (Moreira et al. 2012). These deficits were observed to be similar as that of systemic rotenone administration but more consistent (Saravanan et al. 2005; Moreira et al. 2012). Therefore, the bilateral intranigral infusion model is preferred to model the PD neurodegenerative pattern in the present study.

One of the important aspects to be considered in PD treatment is the relief from the motor and non-motor symptoms. A battery of neurobehavioral parameters were assessed through Rota rod, Open field and Barnes Maze test to deal with this aspect of the study. The motor co-ordination, balance and grip of the animal was measured in the Rota rod test as the animal was positioned on the rotating rod. The gradual decline in the latency to fall over the entire duration of the study in the rotenone treated group, suggest

induction of progressive motor impairment. The daidzein treatment mitigated this impairment as the animals exhibited an improved latency to fall. They displayed improved grip strength and muscular coordination similar to the standard levodopa and carbidopa. The open field test further confirmed the improvement in the locomotor activity of the animals as compared to the rotenone treated group.

The symptomatology of PD is also comprised of an array of non-motor symptoms apart from the cardinal motor effects. These non-motor symptoms have been reported to be debilitating with a greater negative influence than the motor impairment on the quality of patient's life. One of the most concerning symptoms is the cognitive decline. Though the impairment in cognitive functions become prominent as the disease progress but mild cognitive dysfunction is observed even in the early phase of PD in approximately 25% patients (Li et al. 2013; Schapira et al. 2017). In the present study BMT was utilized to evaluate the impairment in the cognitive skills of the animals due to rotenone treatment and the ameliorative effect of test phytochemicals. BMT assesses the spatial learning and the ability of the animals to retain it in terms of the escape latency presented by the animals. The results of our study evince the impairment in the ability of animals to learn and retain the acquired memory due to bilateral rotenone infusion. These results therefore make bilateral rotenone administration as one of the toxin induced lesion models which can recapitulate the cognition dysfunction along with the other non-motors symptoms as reported in the literature. The results of the study are in conformance with some earlier studies performed by (Moreira et al. 2012). DZ treatment negate the rotenone induced cognitive decline, which was observed to be similar to normal memory retention. This was conspicuous from the ELT decline over the test duration. The results of neurobehavioral analysis therefore demonstrate that the bilateral rotenone infusion was successful in induction of the Parkinsonian symptoms preclinically, recapitulating the symptomatic feature of the disease. Daidzein treatment was found to attenuate the impairment induced by rotenone, therefore successfully alleviating the symptoms.

We further analysed the biochemical and mechanistic basis for the recovery observed by means of determining the effect of DZ on the oxidative stress, inflammation and mitochondrial dysfunction. Mitochondrial respiratory defects have been central to the pathogenic mechanisms responsible for neuronal degeneration in PD. The mitochondrial dysfunction initiating from the complex I inhibition perturbs the ETC activating a chain of events ultimately leading to neuronal death. The reduction in ATP production, the subsequent rise in reactive oxygen species (ROS), increased protein aggregation (α -synuclein) and stimulation of apoptotic pathways resulting in cell death (Betarbet et al. 2000). Accordingly, a distinctive midbrain complex I activity inhibition in the rotenone treated animals was observed after bilateral infusion in SN. This lead to increase in oxidative stress as evident from the decreased levels of cellular anti-oxidant defences i.e. Reduced GSH, SOD and Catalase. This was further confirmed by the rise in MDA indicating the membrane damage due to lipid peroxidation. The ROS inflicted lipid peroxidation, sets in a self-propagating chain reaction disrupting the cellular membrane. ROS is also known to stimulate the pro-inflammatory pathways causing perpetuation of the deleterious environment specifically for the vulnerable midbrain DA neurons. According to the available literature, oxidative stress and neuroinflammation both act as co-conspirators ensuing a vicious cycle in the PD neurodegenerative pathogenesis (Whitton 2007; Taylor et al. 2013). We determined the levels

of TNF- α as a pro-inflammatory marker for cytokine signaling pathways which is released by activated microglia as a response to cellular damage. As anticipated, a rise in the levels of TNF- α was noted in the rotenone treated animals as compared to the control animals therefore confirming the induction of neurotoxic inflammation in the midbrain. The elevation in GFAP immunostaining in midbrain further affirmed this finding. GFAP is considered to be a marker of reactive astrocytes, which leads to upregulation in the expression of TNF- α encoding genes (Sherer et al. 2003; Troncoso-Escudero et al. 2018). The activated mitochondrial dysfunction, oxidative stress and neuroinflammation render the DA neurons susceptible to damage as observed by the depletion of TH positive neurons in SNpc as well as striatum, resulting in a significant neurodegeneration in midbrain. The magnitude of TH immunoreactivity is critical as it equates to the functionality of DA neurons in these areas (Halliday et al. 1990; Fukuda et al. 1999). Since TH acts a rate limiting enzyme in the production of Dopamine from precursor L-DOPA (Daubner et al. 2011), the degeneration of these neurons plays a major role in the motor disability observed after rotenone treatment.

According to the literature survey, DZ has been reported to possess anti-oxidant and anti-inflammatory activity (Schreihöfer 2015; Morissette et al. 2018). The biochemical analysis substantiated the anti-oxidant and anti-inflammatory potential of DZ. The DZ treatment was able to maintain the levels of the protective cellular antioxidant machinery similar to the control group and therefore restrict the lipid peroxidation. The TNF- α levels was found to be decreased in the mid-brain tissues with a lower GFAP level. This effect could be due to the prevention of complex I inhibition by rotenone. These protective effects are ultimately translated in to prominent conservation of the TH positive DA neurons both in midbrain implying neuroprotection conferred by DZ.

To elucidate the mechanism for the observed neuroprotection we focussed on mitochondria and its dysfunction since it marks an early event in neurodegeneration. The susceptibility of neurons to mitochondrial dysfunction is attributed to the neurons high energy demand, strict regulation of ATP-dependent processes and dearth of antioxidant defenses. These factors make them highly dependent on the status of mitochondrial homeostasis (Adibhatla and Hatcher 2010). The significance of mitochondrial dysfunction is further reiterated with the discovery of defects in genes associated with mitochondrial homeostasis and quality control pathways such as mutations in PINK1 and parkin (Clark et al. 2006). These genetic defects lead to an imbalance between mitochondrial biogenesis and disposal of dysfunctional mitochondria resulting in accumulation of dysfunctional mitochondria (Schapira and Gegg 2011; Maiti et al. 2017; Park et al. 2018). Therefore, a therapeutic strategy focusing on rectifying this dysfunction with the promotion of mitochondrial biogenesis could prove to be beneficial. Mitochondrial biogenesis (MB) is a complex transcriptional program of nuclear and mitochondrial encoded genes resulting in the repair, division as well as growth of pre-existing mitochondria. It is associated with boosting the cellular bioenergetic capacity by increasing the cellular mitochondrial components (Ventura-Clapier et al. 2008). The stimulation in mitochondrial biogenesis results in an enriched wild type mitochondrial population and will help in compensating the OXPHOS deficit associated with neurodegeneration. This shift therefore can lead to a healthy mitochondrial population thus optimizing the respiratory and regulatory function in the cell and preventing the further

degenerative pathological processes (Moraes 2009; Wenz et al. 2010; Uittenbogaard and Chiaramello 2014).

A detailed literature survey for daidzein reveals that it possesses the propensity to boost the MB. This is evident by the studies reporting the upregulation of SIRT1, Peroxisome proliferator-activated receptor gamma (PPAR γ), peroxisome proliferator-activated receptor γ coactivator (PGC)-1 α and mitochondrial transcription factor A (Tfam) expression by DZ (Rasbach and Schnellmann 2008; Yoshino et al. 2015). It further enhances the expression of OXPHOS genes such as Cox1, Cytochrome B (Cytb), ATP synthase subunit 5 (Atp5) thus increasing the mitochondrial DNA content. DZ is reported to modulate MB through Nuclear respiratory factor (NRF)-PGC1 α network through a SIRT1-dependent pathway (Yoshino et al. 2015). Considering the aforementioned reports, daidzein is considered as a potential candidate to confer a neuroprotective effect in PD mediating its activity through mitochondrial biogenesis. We evaluated the expression of a MB marker namely mitochondrial COX1 (MtCO-1) to assess its activation. COX1 (MtCO-1) is a downstream target of PGC1 α and a polypeptide encoded by mitochondrial DNA in the MB process (Heilbronn et al. 2007; Novin et al. 2015; Chuang et al. 2019). Our results corroborate with the literature as it demonstrates a marked upregulation of COX 1 therefore confirming the stimulation of MB in treated rats. Interestingly, rotenone treatment showed a significant downregulation of COX 1, suggesting disruption of mitochondria ultimately responsible for bioenergetic imbalance followed by neuronal damage. The complex I activity evident in the respective groups justify these results therefore attesting the contribution of MB to the recovery of mitochondrial function by DZ.

Conclusion

In conclusion, daidzein was successful in mitigating the pathogenic effects and parkinsonian symptoms induced due to rotenone administration. This neuroprotective effect was a collective consequence of the ability of daidzein to prevent the complex I inhibition and therefore attenuate the oxidative stress and inflammation. The recovery could be mechanistically credited to the upregulation in the mitochondrial biogenesis which served to maintain the mitochondrial homeostasis and therefore neuronal health. Considering all the above results, daidzein can be considered as a potential candidate in the search for better therapeutic option in the treatment of PD.

Declarations

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Authors' contributions:

Vaibhavi Peshattiwar designed and performed animal studies.

Vaibhavi Peshattiwar, Suraj Muke, Aakruti Kaikini and Sneha Bagle helped in performing animal studies, biochemical, histological, and TNF- α analysis and analysed the data.

Vaibhavi Peshattiwar and Vikas Dighe performed the immunohistochemistry and RT-PCR analysis.

Sadhana Sathaye designed the research and supervised the project.

Vaibhavi Peshattiwar, Sneha Bagle, Aakruti Kaikini and Sadhana Sathaye contributed to writing and editing of the manuscript

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Figures

Figure 1

Schematic illustration of experimental design

Figure 2

Rota rod performance in different study groups on Day 0, 3, 15 and 30. Data expressed as mean $\pm$ SEM. $n=10$. ### $P < 0.001$ compared with Control group and *** $P < 0.001$ compared with Rotenone treatment group. Data analysed using one-way ANOVA followed by Dunnett's test as a post-ANOVA test

Figure 3

Open field test performance in different study groups on Day 0, 3, 2 and 29. Data expressed as mean $\pm$ SEM. $n=10$. ### $P < 0.001$ compared with Control group and *** $P < 0.001$ compared with Rotenone treatment group. Data analysed using one-way ANOVA followed by Dunnett's test as a post-ANOVA test

Figure 4

Barnes maze test performance by different groups performed on day 21 to 27. Data expressed as mean $\pm$ SEM. $n = 10$. # $P < 0.005$ and ## $P < 0.001$ compared with control group using two-way ANOVA followed by post hoc Bonferroni test

Figure 5

Effect of treatment on oxidative stress and inflammatory parameters in different study groups. Data expressed as mean $\pm$ SEM. n=6. ### P< 0.001 compared with Control group * P <0.05, ** P <0.01 and *** P < 0.001 compared with Rotenone treatment group. Data analysed by using One-way ANOVA followed by Dunnett's test as a post-ANOVA test

Figure 6

(a) Effect of treatment on mitochondria complex I in mid brain of different study groups. Data expressed as mean $\pm$ SEM. $n=6$. ### $p < 0.001$ compared with Control group * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with Rotenone treatment group. Data analyzed by using Two-way ANOVA followed by Dunnett's test as a post-ANOVA test. (b) Effect of treatment on the expression of MtCO1/COX1 in mid-brains of different study groups. Data expressed as mean $\pm$ SEM. ### $p < 0.001$ compared with Control group * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with Rotenone treatment group. Data analyzed by using Two-way ANOVA followed by Tuckey's test as a post-ANOVA test

Figure 7

Effect of treatment on TH immunohistochemistry in (a) substantia nigra and (b) striatum of animals of different groups. Data expressed as mean $\pm$ SEM. ### $p < 0.001$ compared with Control group * $p < 0.05$,

****p < 0.01 and ***p < 0.001 compared with Rotenone treatment group. Data analyzed by using Two-way ANOVA followed by Dunnett's test as a post-ANOVA test**

Figure 8

Effect of treatment on GFAP immunochemistry in different study groups. Data expressed as mean $\pm$ SEM. ### p < 0.001 compared with Control group *p < 0.05, **p < 0.01 and ***p < 0.001 compared with Rotenone treatment group. Data analyzed by using Two-way ANOVA followed by Dunnett's test as a post-ANOVA test