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ORIGINAL ARTICLE

Neuroprotective Effect of Neferine, an Alkaloid against the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine Induced Parkinson's Disease Mouse Model

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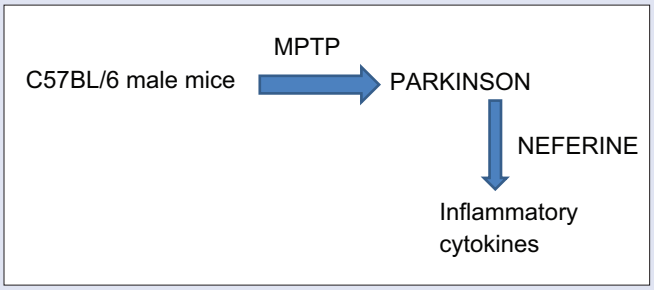
ABSTRACT

Background: Parkinson's disease (PD) is the second most chronic neurodegenerative disorders affecting the age-old peoples worldwide. Therefore, in the present study, we assessed the neuroprotective effect of neferine against the progressive Parkinson's induced mouse model. Mice were induced PD by treating them with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin selectively disrupts the dopaminergic neurons and then treated with neferine, a plant alkaloid. **Materials and Methods:** The neuroprotectant effect of neferine against MPTP-induced Parkinson's was assessed with behavioral analysis such as grid test and stride length measurement test, and the motor coordination was confirmed with rota rod test. Further, to assess the anti-inflammatory property of neferine, the levels of pro-inflammatory cytokines tumor necrosis factor-α, interleukin (IL)-1β and IL-6 were estimated. **Results:** To confirm the neuroprotectant effect of neferine, the levels of dopamine and the protein expression of inducible nitric oxide synthase, cylcooxygenase-2, rate-limiting enzyme of dopamine synthesis tyrosine hydroxylase in the substantia nigra tissue of control and experimental mice were evaluated. Our overall results authentically prove that neferine acts an anti-inflammatory agent decreased the levels of pro-inflammatory cytokines and increased the dopamine levels in substantia nigra, thereby protecting the mice from MPTP-induced Parkinsonism. **Conclusion:** The behavioral analysis also confirmed neferine as a persuasive neuroprotectant with nil side effects and can be prescribed as a drug to treat Parkinson's with subject to further trials.

Key words: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinson's disease mice model, behavior analysis, neferine, neurodegenerative diseases, neuroinflammation, neuroprotectant

SUMMARY

- Pro-inflammatory cytokines tumor necrosis factor-α, interleukin (IL)-1β, IL-2, IL-6 and transforming growth factor-β1 levels were reported to be elevated in the striatum, substantia nigra and cerebrospinal fluid of Parkinson's disease patients
- The behavioral analysis with grid test, stride length and rota rod authentically confirms the neuroprotective effect of neferine against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine treatment.



Abbreviations used: PD: Parkinson's disease; MPTP: 1-methyl-4-phenyl-1,2, 3,6-tetrahydropyridine; COMT: Catechol-o-methyl transferase.

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INTRODUCTION

Parkinson's disease (PD), is the second most prevalent neurodegenerative disorders occurring in age old population worldwide.[1] The incidence rate of PD has increased to 3% in aged people between the age group of 80–90 years and 1% between the age group of 65–69 years population.[2] The key pathophysiology of PD is the neurodegeneration of dopaminergic neuron of the substantia nigra, which does not produces any symptom until 80% of dopaminergic neurons are degenerated. The clinical motor symptoms exhibited by Parkinson's patients are bradykinesia, tremor, postural instability, and rigidity, leading to deprived standard of living in PD patients. It also causes cognitive decline, neubehavioral alterations, sleep disorders, and autonomic neuron dysfunction.[3,4]

At present, the drugs prescribed to treat PD either increase the levels of dopamine or act as substitute of dopamine *in vivo*. Leveodopa is the most commonly prescribed drug for PD patients; it acts as dopamine precursor and increases the level of dopamine.[5] The second variety of drugs such as monoamine oxidase B inhibitors and catechol-o-methyl transferase inhibitors decrease the metabolism of dopamine, thereby preventing the depletion of dopamine levels.[6] Both of these drugs on long-term usage render serious side effects, which urge researchers to discover a potent drug to treat PD with nil side effects.

Herbal medicines which are prescribed by traditional Chinese, Ayurvedic, and Siddha to treat various diseases are the potent

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alternative for the allopathic drugs. The research on herbal based drugs are increased drastically in the last decade and global marketing of herbal drugs predicted to be five trillion USD by the year 2020.[7] Phytochemicals and secondary metabolites of the plants play key role in alleviating numerous diseases, one such phytochemical is alkaloids which possess various pharmacological properties such as anticancer, antibacterial, antidiabetic, analgesic, neuroprotectant.[8,9] Neferine, bisbenzylisoquinoline alkaloid isolated from seeds of Nelumbo nucifera reported to possess anti-inflammatory,[10] anticancer[11] and neuroprotective, antidepressant[12] properties.

Therefore, in the present study, we assessed the neuroprotective property of neferine against chronic Parkinson's induced mice model. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced Parkinson mouse model are the widely preferred model of Parkinson induction.[13] Nonhuman primates treated with MPTP shown motor disabilities resembling the symptoms of idiopathic Parkinsonism.[14] Hence, we induced Parkinsonism in mice with MPTP and analyzed effect neferine on the motor coordination, dopaminergic neuron inflammation and dopamine synthesis in PD-induced mice model.

MATERIALS AND METHODS

Chemicals

MPTP (23007-85-4), Neferine (2292-16-2), bovine serum albumin, Tris HCL, and Tween20 were purchased from Sigma Aldrich, USA. Monoclonal primary antibodies were procured from Cell Signaling Technology, USA. HRP conjugated secondary antibodies and Enhanced chemiluminescence kit was obtained from ThermoFischer Scientific, USA. All the other chemicals used in the current are of analytical grade only.

Animals

Young healthy male C57BL/6 mice weighing about 25–30 g were procured from the institutional animal house after approval of institutional ethical committee. All the procedures followed in the present study were clearly presented before the expert committee and got approved by the committee. The animals were acclimatized for 1 week at 12/12 h light dark cycle, 50%–60% humidity, 26°C and fed with standard laboratory mice feed, sterilized reverse osmosis water ad libidum. The animals were handled with utmost care and concern; all possible means were followed to minimize the sufferings of animals.

Parkinson mice model

After the acclimatization period, the mice were induced Parkinsonism by treating them with 30 mg/kg bwt of MPTP for 5 consecutive days. MPTP was injected intrapertionealy, which disrupts the dominergic neurons of substantia nigra and causes Parkinson-like symptoms.

Experimental protocol

Healthy C57BL/6 mice were randomly divided into four groups namely Group I – control, Group II – Parkinson-induced mice (PD); Group III – Parkinson-induced simultaneously neferine treated, and Group IV – drug control. Each group consists of six mice, Group I mice were treated with corn oil intraperitoneally for 14 days, Group II mice were treated with MPTP (30 mg/kg bw) intraperitoneally for 5 consecutive days. Group III mice were treated with MPTP (30 mg/kg bw) for 5 consecutive days and also treated with nefereine (20 mg/kg bw) intraperitonial injection from day 10 to 14 of treatment period. Group IV were treated with nefereine (20 mg/kg bw) intraperitonial injection for 14 days. Animals were euthanized after the treatment period and the blood was collected through heart puncture and the brain was dissected, immediately stored at −4°C for further analysis.

Grid performance test

The motor coordination of the control and experimental mice were assessed 24 h after the last treatment period using grid apparatus which is a horizontal mesh mounted above 20 cm hard surface at an angle of 90°C.[15] The mice were acclimatized for 30 min in the testing environment before the initiation of experiment. The mice were placed on to the grid, once confirmed the mice have grabbed the mesh with all the paws, the mesh was tilted upside down and the number of squares crossed by mice within 30 s was recorded. The experiment was performed by the same individual to avoid false-positive or -negative results due to different animal handlers and the experiment were repeated five times for each mice, grid was cleaned with 70% ethanol in between each experiment. The average fore paw distance was calculated as the total distance covered by the mice divided by the number of steps.

Stride length measurement

The stride length measurement was performed according to the protocol of D'Hooge 1999.[16] The animals were acclimatized to the experimental set up for 3 days before performing the experiment. The apparatus consists of open field connected to a dark wooden box through a runway, the open field and the run way was illuminated with 60 W light. A clean white paper was placed on the run way and the mice was placed on to the open field with the red ink wetted forepaws. The experiment was repeated for three times for each mice, and the stride length between the paws was measured. The apparatus was wiped with 70% ethanol before the initiation of each experiment.

Rota rod performance

The rota rod performance of control and experimental mice was carried with rota rod apparatus equipped with different rotating speed and digital falling sensors (NSAW, India). The mouse was placed on the 25 mm diameter stationary and 5 rpm rotating rod for a training period of 3 min for 3 consecutive days. The acceleration of rod was increased 5, 10, and 15 rpm, the average withstanding time of mice on the rotating rod was measured. Each mouse was placed on to the rotating rod for 3 min, and the apparatus was cleaned with 70% before initiation of each experiments. All the behavioral analyses were performed by the same researcher at same environment and only during the light cycle.

Dopamine estimation

Substantia nigra of the control and experimental mice were dissected, weighed, and homogenized with 100 µl of homogenizing buffer consisting of 4 mm sodium metabisulfite, 0.01 N hydrochloric acid, and 0.15 mM EDTA. The homogenized mixture was subjected to centrifugation at 12,000 rpm for 15 min and the supernatant was collected for the analysis of dopamine content using commercially available ELISA kit (Eagle Biosciences, NH, USA). The assay was performed according to the manufacturer's protocol and the OD values of the samples were measured at 450 nm within 15 min after the addition of stop solution. The results were expressed as µg/g protein of tissue sample.

Pro-inflammatory cytokine estimation

The pro-inflammatory cytokine levels in the control and experimental rats were assessed with commercially available ELISA kits. The Substantia nigra tissue of control, PD-induced, PD-induced and subsequently neferine-treated, and neferine alone-treated mice were homogenized with RIPA buffer and subjected to centrifugation at 12,000 rpm for 15 min. The supernatant was subjected to the estimation of tumor necrosis factor-α (TNF-α) (MBS355371), interleukin (IL) 1β (MBS774854), IL-6 (MBS2508516), MyBiosource, USA, as per the

manufacturer's protocol. The tissue lysates were added on to the ELISA plates coated with anti-TNF-α, IL-1β, and IL-6 followed by the addition of biotin conjugated antibodies. The color change produced by the TMB substrates was measured at the absorbance of 450 nm and the levels of TNF-α, IL-1β, and IL-6 were measured using the standard curve drawn with the values concentrations of standard test samples.

Immunoblotting analysis

50 mg of Substantia nigra tissue of control and experimental animals was homogenized with 0.5 ml RIPA buffer in tissue homogenizer. The tissue was homogenized gently in the presence of ice cubes to avoid protein degradation. The tissue lysates were then centrifuged at 12,000 rpm of 15 min and supernatant was collected, subjected to protein estimation using Bradford reagent. 40 µg of protein sample from each group were electrophoresed using the sodium dodecyl-sulfate polyacrylamide gel electrophoresis at 100 mV for 1 h. The separated protein samples were then transferred to the polyvinylidene fluoride membrane and the transferred membrane was blocked with 5% blocking buffer to avoid non-specific antibody binding. After the blocking step, the membranes were rinsed with tris buffered saline and incubated with mouse polyclonal primary antibodies inducible nitric oxide synthase (iNOS) (#2982), cylcooxygenase-2 (COX-2) (#4842), TH (#2792) procured from Cell Signaling Technology, USA at dilution of 1:1000, for a period of overnight at 4°C. After the incubation period the membranes were rinsed with tris buffered saline, tween-tris buffered saline for three times and incubated with HRP conjugated secondary antibody at a dilution of 1:10,000 for 1 h at room temperature. The membranes were assessed for protein bands using enzyme chemiluminescence kit (Thermo Fisher Scientific Inc., USA).

Statistical analysis

The data of both behavioral and biochemical analysis were assessed statistically using the GraphPad Prism software (GraphPad Software, Inc., CA, USA). The data of behavioral analysis were statistically assessed with Student's *t*-test and expressed as mean ± standard error of the mean and the biochemical results were assessed with one-way analysis of variance followed by *post hoc* Student–Newman–Keuls test. $P < 0.05$ was considered statistically significant.

RESULTS

Neuroprotective effect of neferine on Parkinson's disease-induced mice's behavioral performance

The difference in behavior of PD-induced mice and the neferine-treated mice were assessed using the grid performance test [Figure 1a]. The average forepaw distance traveled by the PD-induced mice is drastically decreased compared to the control mice whereas neferine treatment on PD-induced mice significantly increased the average forepaw distance compared to the PD alone induced mice. Neferine alone injected mice crossed same distance as that of the control mice it does not show any significant difference compared to the control mice which implies neferine does not possess any sedative effects.

Neuroprotective effect of neferine on Parkinson's disease-induced mice's nigrostriatal dysfunction

To assess the neuroprotective effect of nefereine on nigrostriatal neurons, a novel behavioral analysis stride length performance test was done [Figure 1b]. The mean forepaw stride length of PD-induced mice (3.51 ± 1.25 cm) was comparatively lower than the control mice (7.21 ± 2.1 cm). Nefereine treatment decreased the nigrostiatal dysfunction thereby increased the forepaw stride length to 5.80 ± 1.57 cm. No significant change was observed in the mean forepaw stride length between the neferine alone-treated mice (6.95 ± 3.2 cm) and the control mice.

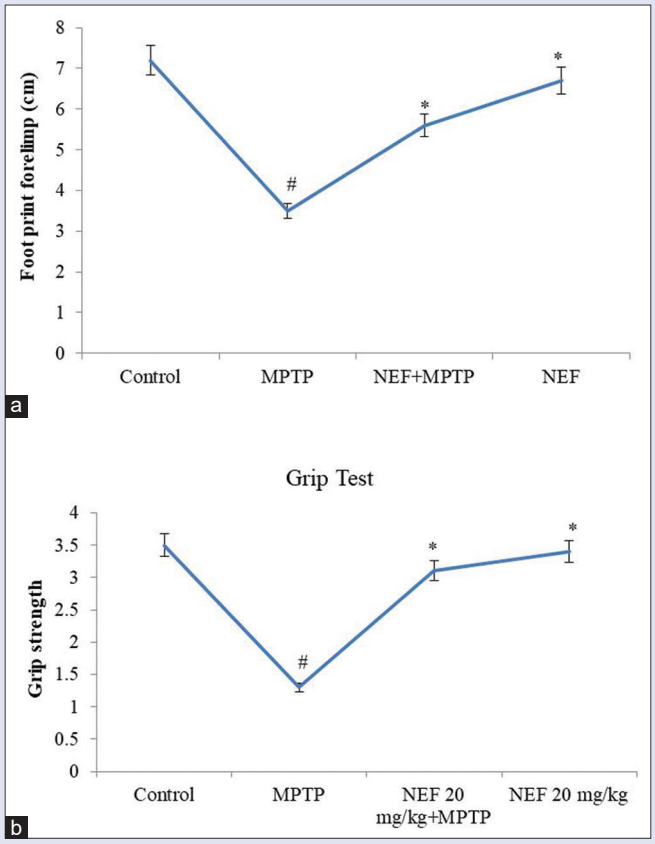

Figure 1: Neuroprotective effect of alkaloid neferine on Parkinson's disease-induced mice nigrostriatal dysfunction. The mice were subjected to Parkinson's disease induction with 1-methyl-4-phenyl-1,2, 3,6-tetrahydropyridine with 1-methyl-4-phenyl-1,2, 3,6-tetrahydropyridine (30 mg/kg bw) intraperitoneally for 5 consecutive days, nefereine (20 mg/kg bw) intraperitonial injection was given from day 10 to 14 of treatment period to Parkinson's disease-induced mice and nefereine (20 mg/kg bw) intraperitonial injection alone was administered for 14 days. 24 h after the treatment period the mice were subjected to Grid performance test (a) and Stride length measurement test (b). The behavior pattern of the mice was video tapped and assessed for the average forepaw distance travelled. The data were statistically analyzed with Student's *t*-test using GraphPad Prism software and expressed as mean ± standard error of the mean. $P < 0.05$ was considered to statistically significant

Neuroprotective effect of neferine on Parkinson's disease-induced mice's motor coordination

Rota rod behavioral analysis was performed to assess the balance and motor coordination of control and experimental mice. The mice were assessed with different speed of rotation such 5, 10, and 15 rpm. The motor coordination of control and neferine mice were significantly more compared to the PD-induced mice. Even after increasing the speed to 10 and 15 rpm the control and neferine alone-treated mice able to withstand on the rod for longer duration of time such control mice 280 ± 9 s at 10 rpm and 175 ± 12 s at 15 rpm. The neferine alone treated mice rested on the rotating rod till 265 ± 15 s at 10 rpm and 192 ± 10 s at 15 rpm. The motor coordination was drastically decreased in PD-induced mice, the mice cant' able to withstand on the rotating rod more than 155 ± 8 s at

5 rpm [Figure 2a], 127 ± 10 s at 10 rpm [Figure 2b] and 92 ± 14 s at 15 rpm [Figure 2c]. Neferine treatment increased the motor coordination in PD-induced mice and the average time of mice on rota rod was significantly increased to 220 ± 10 s at 10 rpm and 174 ± 12 s at 15 rpm speed.

Neuroprotective effect of neferine on Parkinson's disease-induced mice's dopamine level

The motor cortex synaptic plasticity and motor skill learning is regulated by the neurotransmitter dopamine synthesized by the dopaminergic neurons of substantia nigra. Figure 3 depicts the levels of dopamine in the Substantia nigra tissue of control and experimental mice. The dopamine levels were drastically decreased in the MPTP treated PD-induced mice whereas it is increased with the neferine treatment to PD-induced mice. Compared to the control mice, the neferine alone-treated mice did not show any significant change in their dopamine levels.

Anti-inflammatory effect of neferine on Parkinson's disease-induced mice

Neuroinflammation acts as an initiation factor for dopaminergic neurodegeneration, hence we assessed the levels of pro-inflammatory cytokines (TNFα, IL-1β, and IL-6) in control and experimental mice [Figure 4]. The levels of all the three cytokines TNFα, IL-1β, and IL-6 were significantly increased in PD-induced mice (8.6 ± 0.02, 13.9 ± 0.06, 8.9 ± 0.04 pg/mg protein) compared to the control mice (5.8 ± 0.05, 8.7 ± 0.02, and 6.3 ± 0.08 pg/mg protein, respectively). Neferine treatment decreased the levels of cytokines TNF-α, IL-1β, and IL-6 to (6.3 ± 0.04, 11.4 ± 0.07, and 7.2 ± 0.02 pg/mg protein) in PD-induced mice and no significant difference was observed in the cytokines TNF-α, IL-1β, and IL-6 levels (5.5 ± 0.03, 8.2 ± 0.05, and 6.1 ± 0.02 pg/mg protein) of neferine alone-treated mice compared to the control mice.

Anti-inflammatory effect of neferine against inflammatory proteins in Parkinson's disease-induced mice

Figure 5 depicts the protein bands of iNOS and COX-2 expression in control and experimental mice. The levels of iNOS and COX-2 were reported to be increased in the patients with Parkinsonism and also in MPTP treated PD-induced mice. Both the levels of iNOS and COX-2 were significantly increased PD-induced mice compared to control mice, whereas neferine treatment on PD-induced mice decreased levels of iNOS and COX-2.

Neuroprotective effect of neferine on rate-limiting enzyme tyrosine hydroxylase in Parkinson's disease-induced mice

MPTP treatment significantly decreased the level of rate limiting enzyme of dopamine synthesis, tyrosine hydroxylase protein expression which is significantly increased with neferine treatment to PD-induced mice. No significant changes were observed between the control and neferince alone treated mice TH protein expression in the Substantia nigra tissue [Figure 5].

DISCUSSION

Neuroinflammation of dopaminergic neurons in the substantia nigra regions is the trait feature of Parkinson induction causing motor and non-motor disturbance.[17] MPTP, neurotoxicant easily crosses the blood brain barrier due to lipophilic nature thereby metabolized to 1-methyl-4-phenylpyridinium by the monoamine oxidase B enzymes present in nondopaminergic neurons. Once metabolized

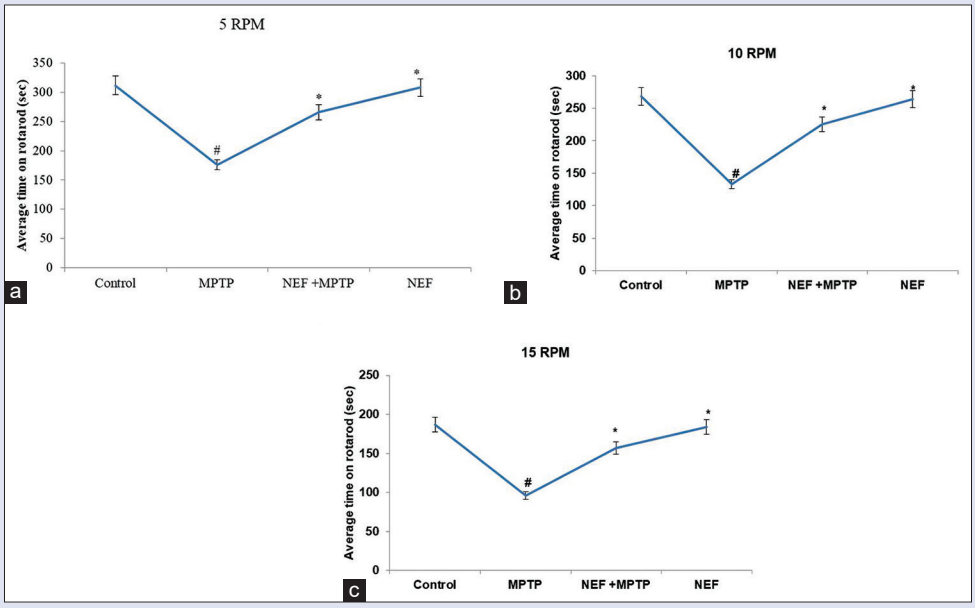

Figure 2: Neuroprotective effect of alkaloid neferine on Parkinson's disease-induced mice motor coordination. The mice were subjected to Parkinson's disease induction with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (30 mg/kg bw) intraperitoneally for 5 consecutive days, nefereine (20 mg/kg bw) intraperitonial injection was given from day 10–14 of treatment period to Parkinson's disease-induced mice and nefereine (20 mg/kg bw) intraperitonial injection alone was administered for 14 days. Last 3 days of the treatment period, the mice were trained to rest on the rotating rod at 5 rpm rotation. 24 h after the last treatment period the mice were subjected to rota rod test at various speed 5 rpm (a), 10 rpm (b) and 15 rpm (c) for 5 min. The behavior pattern of the mice was video tapped and assessed for the average withstand time of each mice on the rotating rod. The data were statistically analyzed with Student's *t*-test using GraphPad Prism software and expressed as mean ± standard error of the mean. $P < 0.05$ was considered to statistically significant

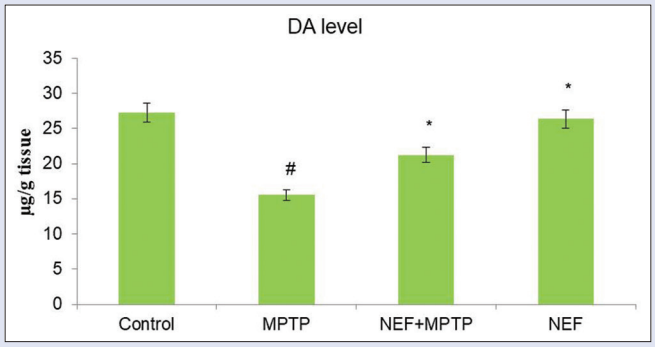

Figure 3: Neuroprotective effect of alkaloid neferine on Parkinson's disease-induced mice dopamine level in the substantia nigra tissue. The mice were subjected to Parkinson's disease induction with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (30 mg/kg bw) intraperitoneally for 5 consecutive days, nefereine (20 mg/kg bw) intraperitonial injection was given from day 10–14 of treatment period to Parkinson's disease-induced mice and nefereine (20 mg/kg bw) intraperitonial injection alone was administered for 14 days. 24 h after the treatment period the mice were euthanized and the substantia nigra was isolated from dissected brain of control and experimental mice. The tissue was lysed with homogenizing buffer and subjected to measurement of dopamine levels using commercially available dopamine ELISA kit. The data were statistically analyzed with one way analysis of variance followed by Student–Newman–Keuls *post hoc* test using GraphPad Prism software and expressed as mean ± standard error of the mean. $P < 0.05$ was considered to statistically significant

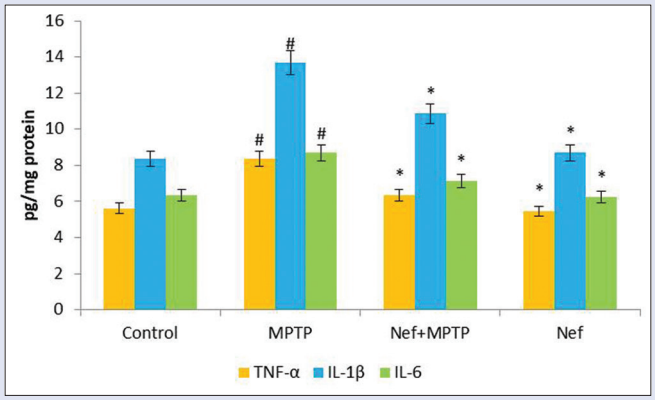

Figure 4: Anti-inflammatory effect of alkaloid neferine on Parkinson's disease-induced mice proinflammatory cytokine levels in the substantia nigra tissue. The mice were subjected to Parkinson's disease induction with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (30 mg/kg bw) intraperitoneally for 5 consecutive days, nefereine (20 mg/kg bw) intraperitonial injection was given from day 10 to 14 of treatment period to Parkinson's disease-induced mice and nefereine (20 mg/kg bw) intraperitonial injection alone was administered for 14 days. 24 h after the treatment period the mice were euthanized and the substantia nigra was isolated from dissected brain of control and experimental mice. The tissue was lysed with homogenizing buffer and subjected to measurement of cytokines tumor necrosis factor-α, interleukin-1β and interleukin-6 levels using commercially available ELISA kit. The data were statistically analyzed with one way analysis of variance followed by Student–Newman–Keuls *post hoc* test using GraphPad Prism software and expressed as mean ± standard error of the mean. $P < 0.05$ was considered to statistically significant

the 1-methyl-4-phenylpyridinium a more potent neurotoxicant selectively reaches the dopaminergic neurons through the dopamine plasma membrane transporter resembling as substrate of DAT.[13] This leads to neuroinflammation and depletion of dopamine levels which resembles the classical *in vivo* environment of Parkinson's induction. Anti-inflammatory drugs such as minocycline aspirin, celecoxib and ibuprofen alleviates the dopaminergic neuroinflammation and increased the dopamine levels in MPTP-induced mouse and 6-OHDA induced rat PD models.[18-21] In the present study, neferine, alkaloid effectively increased the levels of dopamine in PD-induced mice this may be due to the antagonistic effect of neferine against DAT which prevented the binding of 1-methyl-4-phenylpyridinium to DAT thereby preventing the dopaminergic neurons from 1-methyl-4-phenylpyridinium induced neuroinflammation.

Pro-inflammatory cytokines TNF-α, IL-1β, IL-2, IL-6, and transforming growth factor-β1 levels were reported to be elevated in the striatum, substantia nigra and cerebrospinal fluid of PD patients. The cytokines levels were significantly increased in CSF and nigrostriatal regions of individuals with PD relative to age-matched healthy controls.[22-24] The patients with elevated levels of cytokines IL-6 and IL-10 were more prone to Parkinson induction.[25,26] Neurtoxicant administration causes microglia activation which subsequently amplifies the neuroinflammation[27] in substantia nigra region through activated astrocytes. The levels of TNF-α and IL-6 were increased *in vitro* astrocyte cells treated with α-synuclein.[28] In the current study also the MPTP treatment increased the levels of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 and the neferine treatment significantly decreased the levels of pro-inflammatory cytokines. Neferine protected the microglia cells and astrocytes from the excessive activation thereby prevented the dopaminergic from neuroinflammation.

Increased levels of enzymes iNOS and NADPH-oxidase were observed only in the activated glial cells of PD patients and MPTP treated mouse model,[29,30] whereas it is completely dormant in normal individuals. Activated glial cells create oxidative stress through the generation of reactive oxygen and nitrogen species thereby induces neurodegeneration in substantia nigra. Elevated levels of proinflammatory cytokines TNF-α, IL-6 and iNOS after LPS treatment were observed in parkin-null mice compared to the normal mice, which implies parkin protein is regulates the inflammatory cytokines in PD patients.[31] COX-2 is yet another important inflammatory protein highly expressed in the PD patients and also in the MPTP treated PD mice model.[32,33] Targeting the inflammatory protein iNOS and COX-2 may be effectively decreasing the dopamine neurons inflammation. Our drug neferine had persuasively inhibited the protein expression of both iNOS and COX-2 in the substantia nigra tissue of PD-induced mice. This confirms the anti-inflammatory property of neferine against the MPTP induced neuroinflammation.

The effect of neferine on dopamine synthesis was assessed by estimating trysoine hydroxylase, the rate limiting enzyme of catecholamine synthesis. MPTP drastically decreased the levels of tyrosine hydroxylase protein expression, whereas it is increased in neferine-treated mice. Our results correlates with the previous studies were the MPTP treatment decreased the TH-positive cells and fibers in cats striaturm[34] and mice.[35] Grid test and stride length measurement were performed to assess the motor skill in control and experimental mice. PD-induced mice shown decreased average forepaw distance travelled whereas it is increased with the neferine treatment. Salicylic acid, a nonsteroidal anti-inflammatory drug effectively alleviates the MPTP induced behavioral changes in mice[36] and also dihydromyricetin, a natural flavanoid treatment increased the locomotor skills of MPTP treated mice. This correlates with our results neferine increased the motor skills and the motor coordination in mice

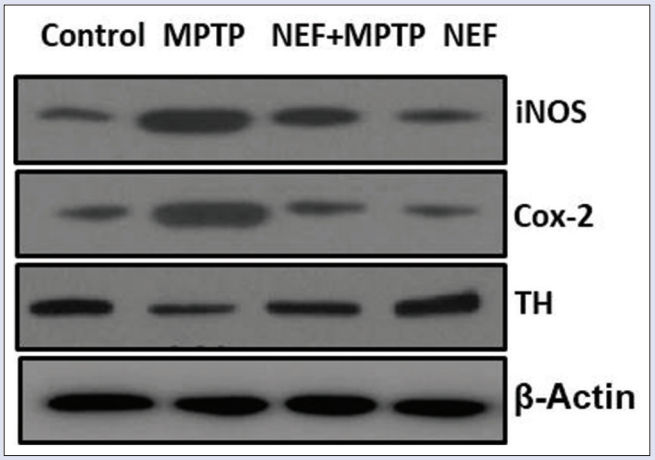

Figure 5: Neuroprotective effect of alkaloid neferine on inducible nitric oxide synthase, cylcooxygenase-2 and TH protein expression in the substantia nigra tissue of Parkinson's disease-induced mice. The mice were subjected to Parkinson's disease induction with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridinewith1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (30 mg/kg bw) intraperitoneally for 5 consecutive days, nefereine (20 mg/kg bw) intraperitonial injection was given from day 10 to 14 of treatment period to Parkinson's disease-induced mice and nefereine (20 mg/kg bw) intraperitonial injection alone was administered for 14 days. 24 h after the treatment period the mice were euthanized and the substantia nigra was isolated from dissected brain of control and experimental mice. The tissue was lysed with RIPA buffer, centrifuged at 1200 rpm for 15 min and the supernatant was subjected to protein estimation. 40 μg of total protein from control and experimental mice samples were subjected to electrophoresis and immublotting analysis with specific proteins inducible nitric oxide synthase, cylcooxygenase-2 and TH protein. The protein bands were visualized using enzyme chemiluminescence kit and representative image were depicted

which is evidenced with rota rod test performance. Thus neferine, eventually inhibited the neuroinflammation induced by MPTP and protected the mice from locomotor impairment.

CONCLUSION

We assessed the neuroprotective effect of alkaloid neferine present in the Chinese herbal medicinal plant *Nelumbo nucifera* on Parkinson induced mice. Our results confirmed the induction of PD through MPTP treatment and neferine significantly protect the PD-induced mice via increasing the levels of dopamine and tyrosine hydorxylase protein expression. It also decreased the levels of proinflammtory cytokines TNF-α, IL-1β, IL-6, iNOS and COX-2 expression in substantia nigra of PD-induced mice. The behavioural analysis with grid test, stride length and rota rod authentically confirms the neuroprotective effect of neferine against MPTP treatment. Overall our findings proves that neferine, a potent phyotchemical can be prescribed as drug to treat PD.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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