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Amelioration of CNS Toxicities of L-Dopa in Experimental Models of Parkinson's disease by Concurrent Treatment with *Tinospora cordifolia*

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Abstract

Parkinson's disease is the second most common neurodegenerative disease, primarily affecting people of ages over 45-55 years, although young adults and even children can also be affected. The gold standard drug for the treatment of Parkinson's disease is L-DOPA, but various studies have proved that the treatment with L-DOPA leads to the death of surviving dopaminergic neurons in the CNS[1]. Hence we have approached to counteract the toxicities of L-DOPA therapy by co-administration of *Tinospora cordifolia* crude powder. On the zero day each animals were given with an intraperitoneal (ip) injection of 1-Methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) (20mg/kg) and after 48 hours the animals were treated with L-DOPA or L-DOPA plus *Tinospora cordifolia* crude powder upto 30 days. At the end of the study period we were evaluated the level of anxiety, grip strength and mitochondrial complex-I activity. The results revealed that the co-administration of *Tinospora cordifolia* crude powder protected the dopaminergic neurons when compared with Sham operated control group. In conclusion, we would like to state that the treatment with *Tinospora cordifolia* crude powder could reduce the toxicities of L-DOPA therapy for Parkinson's disease.

Keywords: Parkinson's disease, CNS toxicities, L-DOPA, *Tinospora cordifolia*, MPTP

1. Introduction

Parkinson's disease (PD) is the most common neurologically based movement disorder, clinically diagnosed by the presence of bradykinesia, postural instability, resting tremor and rigidity[2]. PD occurs when a group of cells in an area of the brain called the substantia nigra (SN) begin to malfunction and die[2].

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These cells in the SN produce a chemical called dopamine. Dopamine is a neurotransmitter, or chemical messenger, that sends information to the parts of the brain that control movement and coordination. When a person has Parkinson's disease, their dopamine-producing cells begin to die and the amount of dopamine produced in the brain decreases[2]. As we enter the new century, Parkinson's disease ranks among the most common late life neurodegenerative diseases, affecting approximately 1.5% to 2.0% of the population older than age 60[3].

The causes are still largely unknown. Current thinking is that major gene mutations cause only a small proportion of all cases and that in most cases; non-genetic factors play a part, probably in interaction with susceptibility genes. Numerous epidemiological studies have been done to identify such non-genetic risk factors, but most were small and methodologically limited[4, 5].

Medications can help manage problems with walking, movement and tremor by increasing the brain's supply of dopamine. The most effective Parkinson's drug is L-DOPA, it passes into the brain and is converted to dopamine. L-DOPA is combined with carbidopa to create the combination drug Sinemet in Europe and L-DOPA is combined with a similar substance, benserazide[6].

In prolonged L-DOPA therapy, the apparent buffering capacity is lost and the patient’s motor state may fluctuate dramatically with each dose of the drug, a common problem is the development of wearing off phenomenon: each dose of L-DOPA affectively improves mobility for a period of time, about 1 or 2 hours, but rigidity and akinesia return rapidly at the end of dosing interval[6]. Increasing the dose and frequency of administration can improve this situation, but this often is limited by the development of dyskinesias, excessive and abnormal involuntary movements[7]. Dyskinesias are observed most often when the plasma L-DOPA concentration is high although in some individuals dyskinesia or dystonia may be triggered when the level is rising or falling. These movements can be as uncomfortable and disabling as the rigidity and akinesia of PD.

In the later stages of PD, patients may fluctuate rapidly between being "off", having no beneficial effects from their medications and being "on" but with disabling dyskinesias, a situation called on/off phenomenon[6]. In addition to motor fluctuations, several other adverse effects are observed after prolonged L-DOPA treatment. A common one is the induction of hallucinations and confusion, particularly common in the elderly. Conventional anti psychotic agents such as phenothiazines are effective in L-DOPA induced psychosis but may cause worsening of Parkinsonism through their actions at the dopamine D_2 receptor [3, 8, 9]. Facial tics, grimacing and mild anxiety, nightmares etc. to severe depression, mania these are also some common side effects of L-DOPA therapy[6].

L-DOPA can generate free radicals during its own oxidation as well as during oxidative metabolism of its product, dopamine. Thus, it appears rational to propose that an excessive quantity of free radicals is generated, and this may be one of the factors which contribute to the side-effects of L-DOPA therapy[1]. Therefore, it is possible that supplementation with appropriate multiple antioxidants may improve the efficacy of L-DOPA therapy[1]. With these supporting evidences it is clear that the L-DOPA toxicity can be attenuated by co-administration of one good antioxidant or a drug which can facilitate the actions and activities of mitochondrial complex-I which is an integral component in Parkinson’s disease [1]. *Tinospora cordifolia* (TC), family: Menispermaceae, has been extensively studied and reported to have potent antioxidant activity[10], literatures report that, if taken regularly in high doses; it has no major side effect and toxicity[10] and it seems TC may bear a potential use in neurodegenerative disease affecting the cerebral neurons[11].

The active adaptogenic constituents present in TC are diterpene compounds including tinosporone, tinosporic acid, cordifolisides A to E, syringen, the yellow alkaloid, berberine, giloin, crude giloininand, a glucosidal bitter principle as well as polysaccharides, including arabinogalactan polysaccharide[12]. Among these berberine ameliorated renal dysfunction in streptozotocin-induced diabetic rats, which was accompanied by inhibition of renal aldose reductase and reduction of oxidative stress[13]. Anti-tumor promoting action of berberine is attributed to its antioxidant property[14]. In the view of above points, we selected *Tinospora cordifolia* crude powder (TCCP) which is wide reported for potent antioxidant[10] and free radical scavenging activities for reducing the toxicities of L-DOPA therapy in experimental PD.

2. Materials and methods

Plant material

Tinospora cordifolia (TC), family: Menispermaceae, were collected from local areas of Coimbatore district, Tamilnadu. The collected areal parts of TC were authenticated from Botanical survey of India, Agriculture University, Coimbatore, Tamilnadu, India. For further reference a voucher specimen has been deposited at J. S. S. College of Pharmacy herbarium, ootacamund, India. *Herbarium accession no*: JSSCP/ P Cog/ 137.

Chemicals

The chemicals which were used for the present study were procured from Sd-Fine Chemicals Mumbai, Sigma Aldrich USA, Loba chemicals Mumbai, Merk chemicals Mumbai.

Preparation of plant material[15]

Aerial parts were cut in to small pieces and dried in sun light. Then they were powdered carefully by using mechanical blender. The powdered drug was finely sieved and kept in air tight container. A further unique advantage of *Tinospora cordifolia* is that it is effective when given orally[16]. So we have selected oral route for the administration of TCCP with 0.3% w/v of carboxy methyl cellulose (CMC) solution as solvent through oral gavage tube.

HPTLC Standardization of Tinospora cordifolia using Tinosporaside[17]

Around 20g of air dried sample was ground to pass through 20 mesh SS sieve and 5g from it was accurately weiged and refluxed with 50ml of methanol about 2h. The resulting solution was filtered, concentrated under vaccum, redissolved in methanol and the final volume was made up to 50 ml. This solution was used for HPTLC analysis.

A camag HPTLC system equipped with a sample applicator Linomat IV, twin rough plate development chamber, TLC scanner III and integration software CATS 4.0 was used. An aluminum plate (10X10 cm) precoated with silica gel 60F 254 (E. Merck) was used as an adsorbent, toluene, acetone and water in the ratio of 5: 15: 1 were used as a mobile phase. The solvent was allowed to run up to 80 mm and the chromatograms were scanned at 220 nm. A 0.5 mg/ml solution of Tinosporaside – reference standard was prepared in methanol as a stock solution. The test solution was shaken well and 15 µl was applied on a TLC plate along with 1, 2, 4, 8 and 16 µl of standard Tinosporaside, likewise three such plates were prepared.

The plates were developed up to 80 mm under chamber saturation condition. After air drying the solvent, the plates were scanned at 220 nm in UV reflectance mode. The amount of Tinosporaside present was determined using the calibration curve plotted between concentration and area of standard. The regression equation was found to be, Y = 7.087X + 107.744 with correlation coefficient of 0.9911. The content of Tinosporaside were quantified and percentage recoveries were calculated (Table 1). By this method the Rf of Tinosporaside was about 0.58. The content of Tinosporaside was found to be 0.05% w/w in sample.

3. Acute toxicity study of TCCP (OECD guide line: 423)[18]

Female Wister rats of weight (180-220g) were taken for the study and kept for overnight fasting. Next day, body weight was taken and TCCP was administered orally at a dose of 2000 mg/kg in 0.3% CMC. Then the animals were observed for mortality and morbidity at 0, 1/2, 1, 2, 4, 6, 8, 12, and 24hours.
Feed was given to the animals after 4 hours of dosing and body weight was checked 6 hours after dosing. Morbidity like convulsions, tremors, grip strength and pupil dilatation were observed. The animals were observed twice daily for 14 days and body weight was taken. The same experiment was repeated once again on 3 female rats (preferably female) as there was no observable clinical toxicity for the animals on the phase 1 study. From acute toxicity study, 200 mg/kg (1/10 of tested dose) of TCCP was selected as dose[11].

Animals

Healthy, adult Wister rats of both sexes (180-220g) were obtained from central animal house facility from J. S. S. College of pharmacy, Tamilnadu, India. Animals were cared for in accordance with guiding principles for the care and use of Animals approved by committee for purpose and control for the supervision and experimentation on animals and Institutional Animal Ethics Committee.*CPCSEA approval number:* JSSCP/ IAEC/ M. PHARM/ PH. COLOGY/ 09/ 2008-09.

Animals were divided into four groups of three male and three female rats in each group.
Group I: Control (normal) group
Group II: Sham operated control (MPTP treated) group
Group III: MPTP + L- DOPA (9 mg/kg) treated group
Group IV: MPTP + L – DOPA + TCCP (200mg /kg) treated group

Induction of Parkinsonism by MPTP[19, 20, 21]

On zero day each animal were given an intraperitoneal (ip) injection of MPTP (20 mg/kg) in normal saline. MPTP (1-Methyl-4-phenyl-1,2,3,6-tertahydropyridine) is a neurotoxin, which after absorption converted to MPP^+ radical which specifically degenerate dopamine-producing neurons in the SN a part of a mid brain. Due to degeneration of dopaminergic neurons the amount of dopamine production will reduce and leads to Parkinsonism. Then forty-eight hours after the induction of Parkinsonism the animals were treated orally with L- DOPA or L-DOPA plus TCCP at 09.00 hours up to 30 days, 0.3% w/v of carboxy methyl cellulose (CMC) solution was used as vehicle.

4. Pharmacological Evaluation

4.1. **Anti-anxiety activity**[22]

The elevated plus maze was used to evaluate the anti-anxiety effect in animals. The apparatus consists of four compartments, two open and two enclosed compartments. After placing animals individually in the centre of the maze, head facing towards the open arm, the stop watch was started and noted down the following parameters for 5 min for each animal. First preference of animal to open or enclosed arm (An arm entry defined as the entry of four paws in to arm). Average time each animal spend in each arm was calculated as, total duration time in arm divided by number of entries. Then compared the percentage preference of animal to open /enclose arm, average time spent in open arm and number of entries in open arm for each group.

4.2. **Muscle grip strength study**[23]

The main symptom of the Parkinson's disease is muscle rigidity. The loss of muscle grip is an indication of muscle rigidity. This effect can be easily studied in animals by using rotarod apparatus. Twenty rpm was selected as an appropriate speed .The animal was placed individually one by one on the rotating rod. Noted the 'fall off time' when animal falls from the rotating rod. Then the fall off time of animal in control and all treated group was compared.

5. Molecular pharmacological evaluation

5.1. **Isolation of mitochondria from rat mid brain**[24]

Tissue was homogenized with a Dounce tissue grinder in mitochondrial isolation buffer (70 mM sucrose, 210 mM mannitol, 5 mM Tris HCl, 1 mM EDTA; pH 7.4) and suspensions were centrifuged at 800 g, 4°C, for 10 min. The supernatant fluids were centrifuged at 13000 g, 4°C, for 10 min, and the pellets were washed with mitochondrial isolation buffer and centrifuged at 13000 g, 4°C, for 10 min to obtain the crude mitochondrial fraction.

5.2. **Estimation of Complex-I activity**[24]

NADH: ubiquinone oxidoreducase (Complex-I) activity was measured in the SN as described in the literature. Brain mitochondria, isolated as above, were lysed by freeze–thawing in hypotonic buffer (25 mM KH_2PO_4, 5 mM $MgCl_2$, pH 7.4). The reaction was initiated by the addition of 50 µg mitochondria to the assay buffer (hypotonic buffer containing 65 µM ubiquinone, 130 µM NADH, 2 µg/ml antimycin A and 2.5 mg/ml defatted bovine serum albumin). The oxidation of NADH by Complex-I was monitored spectrophotometrically at 340 nm for 2 min at 30°C. The activity was monitored for a further 2 min following the addition of rotenone (2 µg/ml). The difference between the rate of oxidation before and after the addition of rotenone was used to calculate Complex-I activity.

6. Statistical analysis

The collected datas were subjected to appropriate statistical tests, one-way ANOVA (Analysis of Variance) followed by Bonferroni multiple comparisons test, nonparametric repeated measures ANOVA followed by Dunnet's multiple comparisons test. P values of less than 0.001 were considered highly significant. The analysis was carried using Graph pad Instat software of version 3.

7. Results

7.1. Effect of L-DOPA and L-DOPA plus TCCP on anxiety behavior

When compared with control group, the MPTP treated group showed more preference to open arm. But, when compared with MPTP treated group, the L-DOPA and L-DOPA plus TCCP treated groups showed less preference to open arm and it indicated that, the treatment with TCCP did not alter the activities or functions of Dopaminergic neurons and could have maintained the dopamine concentration in CNS. The more preference to open arm shows less anxiety, dopamine levels and anxiety are directly proportional.

7.2. Effect of L-DOPA and L-DOPA plus TCCP on muscle grip strength

When compared with control group the retention time was significantly reduced for MPTP treated group. But when compared with MPTP treated group, the L-DOPA and L-DOPA plus TCCP treated groups showed more retention time. The results suggested that retention time or muscle co-ordination was improved by either L-DOPA or L-DOPA plus TCCP treated groups. The data indicated that the treatment with TCCP can facilitate the muscle coordination with some actions on dopaminergic neurons.

7.3. Effect of L-DOPA and L-DOPA plus TCCP on Complex-I activity

The Complex-I activity were estimated from mitochondrial fractions isolated from brain tissue homogenate. When compared with control animals the mitochondrial activity was significantly reduced for MPTP and L-DOPA treated group. But the concurrent treatment with TCCP had significantly retained the Complex-I activity.

8. Discussion

There is growing evidence that oxidative stress and mitochondrial respiratory failures with attendant decrease in energy output are implicated in nigral neuronal death in PD[2]. However it is not known, which cellular elements (neurons or glial cells) are major targets of oxygen-mediated damage. L-DOPA therapy is one of the common therapies for advanced PD. But, the severe side-effects of this therapy appear in about five years[7]. The reasons for this are not known; however, L-DOPA can generate free radicals during its own oxidation as well as during oxidative metabolism of it product, dopamine[25, 26, 27]. Thus, it appears rational to propose that an excessive quantity of free radicals is generated, and this may be one of the factors which contribute to the side-effects of L-DOPA therapy. Selegiline used in combination with

L-DOPA may reduce free radical levels by reducing the oxidative metabolism of dopamine; however, it would not affect the level of free radicals generated by the oxidation of L-DOPA. Earlier studies suggested that supplementation with appropriate multiple antioxidants may improve the efficacy or reduce the toxicity of L-DOPA therapy[26, 27].

With these supporting evidences it is clear that the L-DOPA toxicity can be attenuated by co-administration of one good anti-oxidant or a drug which can facilitate or unaffected the activities of mitochondrial complex-I which deteriorate during L-DOPA therapy. With this concern we have evaluated the effect of TCCP for reducing the toxicities of L-DOPA.

We could evaluate the parameters such as level of anxiety, grip strength and mitochondrial Complex-I activity. The level of anxiety was comparably none for L-DOPA or L-DOPA plus TCCP treated groups. The MPTP treated groups, showed less anxiety and it was comparably reduced when compared with normal control. These findings indicate that, the TCCP treatment did not alter the role or action of L-DOPA in experimental animals. The concept of anti-anxiety test was introduced to assess whether TCCP treatment have any interference with L-DOPA affects action to regain dopamine level in dopamine depleted animals.

From the results of muscle grip strength, it is clear that L-DOPA and L-DOPA plus TCCP did not alter muscle co-ordination. This study suggested and further supports that TCCP treatment did not alter the affects of L-DOPA therapy in experimental PD. Earlier reports evidenced that L-DOPA therapy leads to mitochondrial degeneration and leads to neuronal cell death. At last we estimated the mid brain mitochondrial Complex-I activity and the activity was unaffected by TCCP treatment.

The Complex-I activity in L-DOPA treated group was drastically reduced and it is the valuable finding that L-DOPA treatment could lead to further degenerative effect in surviving dopaminergic neurons. Two consistent biochemical 'signatures' in the SN of idiopathic PD cases are a deficiency of mitochondrial Complex-I and abnormally high levels of free-radical damage[2]. These two events are thought to be interrelated, because inhibition of Complex-I activity can increase free radical production, and increased free radical production impairs Complex-I activity. Studies that have attempted to modify the disease course in PD by reducing free-radical formation have shown relatively modest effects on PD[3]. This is valuable information in this research that the protection of mitochondria by TCCP could solve the toxicity of L-DOPA therapy. From the results it may be clear that the co-administration of TCCP can reduce the toxicities of L-DOPA which is a gold standard drug for Parkinson's treatment.

Apart from this, a different plant Mucuna prurita, family: Fabaceae, seeds contain high concentrations of L-DOPA, it has long been used in traditional ayurvedic Indian medicine for the treatment of Parkinson's disease, but the seed may cause birth defects and has uterine stimulant activity during pregnancy, contraindicated in combination with M AO inhibitors, potentiate androgenic medications, potentiate insulin and antidiabetic medication and prolonged use lead to neurotoxicity as conventional L-DOPA treatment[28].

9. Conclusion

In the conclusion, we would like to state that the treatment with TCCP can reduce the toxicity of L-DOPA therapy for Parkinson's disease. The mitochondrial activity retained by TCCP showed a promising way for the usefulness of TCCP for the treatment of clinical Parkinson's disease. The further pharmacological and clinical investigations are needed to implement it for clinical use.

Table 1. Recovery of Tinosporaside from *Tinospora cordifolia*

Sample	**Amount Fortified (mg/ml)**	**Observed Value (mg/ml)**			**Calculated value (%)**	**Average Recovery s(%)**
TC	0.5	0.997	0.089	0.098	5.10	
TC	0.5	0.451	0.484	0.493	4.65	95%
TC	0.5	0.981	0.933	0.942	2.68	

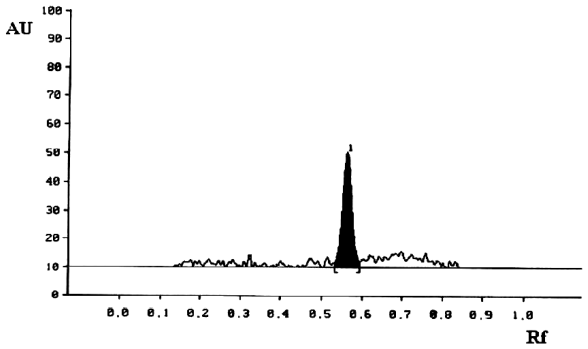

Fig. 1: HPTLC chromatogram of standard Tinosporaside (Rf = 0.58).

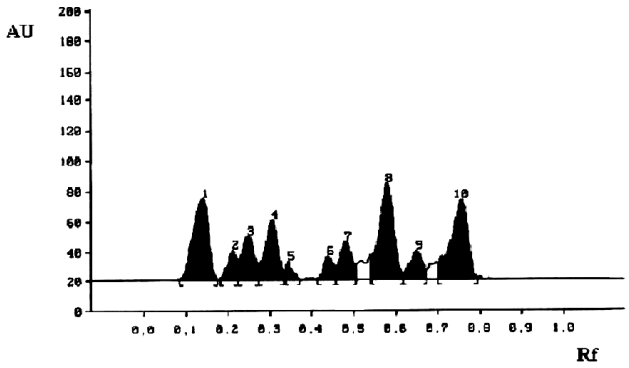

Fig. 2: HPTLC chromatogram of *Tinospora cordifolia* test sample (Rf = 0.58).

Table 2. Effect of L-DOPA and L-DOPA plus TCCP on anxiety behavior

Group	**Average time spent in arm(sec)**		**%Preference to open arm**
	Open arm	**Enclosed arm**	
Control	7.220 ±1.800	58.1122±3.224	10.452±2.0018
Only MPTP treated	14.800 ±1.029	30.7781±2.929	48.220±4.5221***
MPTP + L-dopa	9.812±2.334	59.1195±3.288	12.4187±0.114^{ns}
MPTP + L-dopa + TCCP	8.552±1.770	60.296±3.1102	11.224±1.3890^{ns}

Values are mean ±SEM; n=6 in each group, ***P<0.001 when compared to normal control, ^{ns}P>0.05 when compared to normal control (one-way ANOVA followed by Bonferroni multiple comparisons test)

Table 3. Effect of L-DOPA and L-DOPA plus TCCP on muscle grip strength activity

Group	**Retention time(sec)**
Control	120.442±1.437
Only MPTP treated	10.56±1.678$^{###}$
MPTP + L-dopa	65.974±1.531**
MPTP + L-dopa + TCCP	84.623±1.098***

Values are mean ±SEM; n=6 in each group, ***P<0.001 when compared to MPTP treated group, **P<0.01 when compared to MPTP treated group, $^{###}$P<0.001 when compared to control group, $^{#}$P<0.05 when compared to control group. (one-way ANOVA followed by Bonferroni multiple comparisons test)

Table 4. Effect of L-DOPA and L-DOPA plus TCCP on complex-I activity

Group	**Concentration(nmol/min/mg protein)**
Control	91.6055±1.796
Only MPTP treated	39.5692±2.768$^{###}$
MPTP + L-dopa	24.9425±2.7650**$^{##}$
MPTP + L-dopa + TCCP	89.5217±1.6254***

Values are mean ±SEM; n=6 in each group, ***P<0.001 when compared to MPTP treated group, **P<0.01 when compared to MPTP treated group, $^{###}$P<0.001 when compared to control, $^{##}$P<0.01 when compared to control group. (One-way ANOVA followed by Bonferroni multiple comparisons test)

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