RESEARCH ARTICLE

Study of Nootropic Effect of Improved Nano Formulation Version of Levodopa in a Rat Model of Parkinson's Disease Induced by 6-Hydroxydopamine

Rawal VP*, Gangurde SA

School of Pharmaceutical Sciences, Sandip University, Mahiravani, Nashik, Maharashtra, India.

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ABSTRACT

Background: More studies on Parkinson's disease (PD) symptoms have demonstrated that the condition is multifaceted. PD is associated with cognitive problems & motor impairments, which lower quality of life. 6-hydroxydopamine is a common rodent Parkinsonism neurotoxin. 6-OHDA may damage neurons by increasing oxidative stress from hydroxyl radical production during autoxidation. 6-OHDA and oxidative stress have been shown to impair memory in rats. Thus, 6-OHDA mimics rat Parkinsonian and cognitive decline.

Objective: To construct a stable and easily administered nano-formulation with enhanced bioavailability by adding *Mucuna pruriens* seed phytoactive Levodopa into Nanocochleates.

Materials and Methods: Free L-Dopa, nanoliposomes, and levodopa nanocochleates were tested in 6-OHDA-treated rats with PD on the 14th day following the 6-OHDA injection, open-field, morris-water-maze, and Y-maze tests assessed learning and memory.

Results: Oral LDNC may reduce 6-OHDA-induced catecholamine neurotoxicity, according to this study. In this 6-OHDA-induced PD model, the nano-formulation appeared to restore injured striatal cells *via* antioxidant and DA-enhancing pathways.

Keywords: Parkinson's disease, 6-hydroxydopamine, 6-OHDA, Levodopa, Nanocochleates.

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INTRODUCTION

Parkinson's disease (PD) is a neurological condition that is characterized by the depletion of striatal dopamine and the disruption of the basal ganglia.[1] Rigidity, postural instability, resting tremors, and impaired walking are among the motor symptoms of Parkinson's disease. The basal ganglia, a collection of deep nuclei that are involved in the planning and execution of movement, malfunction as a result of the loss of dopaminergic cells.[2] Cognitive symptoms from minor memory and thinking difficulties to severe dementia can worsen PD. Research shows that almost half of PD patients experience cognitive impairment. More than 20% have severe cognitive impairment. PD can make it hard to focus, recall names, and learn.[3,4] Oxidative stress in PD causes protein, DNA, and lipid metabolism damage, which degenerates DA cells. Because 6-hydroxydopamine (6-OHDA) induces oxidative stress-induced apoptosis, this selective catecholaminergic neurotoxin is commonly utilized to create Parkinson's disease (PD) models for research. Unilateral intrastriatal 6-OHDA administration causes Parkinson 's-like behavioral and metabolic alterations. Neurotoxic 6-OHDA rapidly oxidizes non-enzymatically to hydroxyl radicals, superoxide, and hydrogen peroxide.[5-7]

In preclinical research, antioxidants show promise, but practical use for neurodegenerative diseases is currently impossible. Low bioavailability, instability, restricted transport to tissue, and/or poor antioxidant capability may need large and recurring dosages that are harmful and too high for human usage. Nanoparticle-mediated drug delivery systems may overcome these CNS medicine delivery challenges[8]. This will protect the active substance from oxidation, lowering dosage and maybe enhancing efficacy. The best traditional Parkinson's disease treatment is levodopa. L-3,4-dihydroxy phenylalanine (levodopa) is an aromatic amino acid found in *M. pruriens*. L-dopa and 5-hydroxy tryptamine (serotonin) are *M. pruriens* var. pruriens major therapeutic components. L-dopa is utilized to treat neurodegenerative Parkinson's-disease.

**Author for Correspondence: vaishu.rawal@gmail.com*

Indian traditional medicine treats PD with *M. pruriens* seeds[9]. L-dopa bioavailability is low because the active metabolizes or oxidizes quickly[10,11]. We are investigating nanoparticle-delivered levodopa to solve some of the above issues. We tried to make Nanocochleates, a stable, easily given nano-formulation with L-dopa. Unlike pharmacological analysis, free L-dopa, nanoliposomes, and nanocochleates were tested to determine the best form.

MATERIAL AND METHOD

Material

Drugs and reagents

Levodopa API was obtained from Yucca Enterprises, located in Wadala, Mumbai. Chemicals such as sunflower lecithin were bought from Vitaegen Life Science in Nagpur, Lobe Chemie Pvt. Ltd. in Mumbai provided cholesterol and ethanol, while Sigma Aldrich in Mumbai provided methanol and acetonitrile.

Methodology

Experimental combinations yielded 14 levodopa-containing liposomes. The best of 14 formulations was improved into nanocochleates.

Formulation and evaluation of levodopa loaded nanocochleates by trapping method[12]

The improved formulation and trapping method produced levodopa-loaded nanocochleates. Nanoliposomes were made with cholesterol and sunflower lecithin. The 25 µL (0.1M) calcium chloride solution was dropwise added to each 25 milliliter of optimized nano-liposomal suspension while vortexed. When Nanocochleates were produced, the suspension became turbid. To stabilize Nanocochleates (LDNC) suspensions, they were chilled for six hours or overnight. The LDNC suspension was centrifuged for 15 minutes at 20,000 rpm and -4°C to generate sediment, which was lyophilized and stored. The formulation was evaluated for various pharmaceutical parameters like content, entrapment, release study, size and charge analysis and microscopic.

In- vivo pharmacokinetics of formulation

In-vivo pharmacokinetic (PK) investigations evaluate plasma drug concentration at multiple time points in treated laboratory animals to calculate exposure (AUC). *In-vivo* pharmacokinetic studies examined medication absorption, distribution, metabolism, and excretion. PES RTBCOP/IAEC Clear R-99; Institutional Animal Ethics Committee clearance. The animal house of "P.E.S's-Rajaram And Tarabai Bandekar College Of Pharmacy, Ponda, Goa, used 200 to 250 g albino Wistar rats for the pharmacokinetic investigation. Global Bioresearch Shirwal, Satara supplied the trial animals. Animals went without food or water overnight during the study. To prevent animal loss, each group of ten Wistar rats received 20 mg/kg of pure L-Dopa (in water) and an improved formulation (LDNC) orally. After light anesthesia with isoflurane, the retro-orbital plexus was checked for 0.25 mL of blood. The samples were put in micro-centrifuge tubes with 10 µL of EDTA at the indicated time (15–240 minutes). Plasma was extracted by centrifuging samples at 4000 rpm for 10 minutes. Once drug concentration was determined, 200 µL plasma samples were stored at -20°C. An HPLC examination of the aliquot followed. Plasma L-Dopa levels were measured using a calibration curve. A non-compartmental model was used to assess pharmacokinetic parameters using PK Solver. The mean ± SD was used to represent all values. A significance level of $p < 0.05$ indicated significant differences.

Pharmacological Evaluation

Experimental animals and approval

We bought 250 to 300 g male Wistar rats from the Global Bioresearch Centre in Shirval, Pune. Animals were housed in Polypropylene Cages with a 12-hour light/dark cycle and average environmental conditions of 25 ± 1ºC and 45 to 55% relative humidity. Animals always had tap water and VRK Nutritional Solutions feed pellets from Sangli, Maharashtra. The Institutional-Animal-Ethics-Committee [IAEC]" of the same college mentioned above pharmacokinetics study section approved the study with Approval Number: PES; RTBCOP/IAEC; clear 2021-R-95.

As stated below, animals were randomly divided into Five groups.

Group I control. No injections or drugs were administered to animals.

Group II: vehicle and 6-OHDA injection. About 14 days before and after 6-OHDA injection into right-substantia-nigra, mice received a once-daily oral dosage of normal saline. Group III: Before and after 6-OHDA injection into-right-substantia-nigra, rats received free oral L-dopa (25 mg/kg BW) once daily. Group IV: Levodopa Nanoliposomes (LDNL dosage = 25 mg/kg BW) were orally administered to rats once daily for 14 days before and 14 days after 6-OHDA injection into-right-Substantia-Nigra. Group V: Levodopa Nanocochleates (LDNC dosage = 25 mg/kg BW) were orally administered to rats 14 days before and 14 days after the 6-OHDA injection.

Every rat in every group except the control group received the identical oral medication suspension for 14 days. Rats were injected with 6-OHDA using a stereotaxic apparatus. A Hamilton syringe was utilized to infuse 6-OHDA into the rats for six days after the injury. They assessed their spatial memory with open field, "Morris-Water-Maze Test, and the Novel-Object-Recognition-Test (NORT) at 7 and 14 days following "6-OHDA injection". Hippocampus's surviving neuron density and scavenger enzyme activity (SOD, CAT, and GAPx) were assessed after the rats' brains were removed. MDA levels in the specific area were also measured[13].

For 6-OHDA administration, anesthesia was induced by perfusing 50 mg/kg sodium thiopental i.p. SNpc with 6 µg of 6-OHDA diluted in 2 µL of 0.2%. Ascorbic acid saline using a 30-gauge steel needle to each rat. After surgery, the animals were allowed to recuperate from anesthesia before returning to their cages.

Open Field Test

This experiment explored animal locomotion. On day 14, animals are stressed and their locomotor activity, emotional reaction, and exploratory behavior are observed.[13] Animals are given 6-OHDA and tested outside for memory. For 5 minutes, each rat "crossing" (counting lines or tiles) and "rearing" (resting its front legs on the device's wall) was counted.[14]

Y-Maze Test

The Y-maze measured short-term memory through spontaneous behavior. Y Maze utilized in this study had an equilateral triangle in the center and three 35 cm-long, 25cm-high, 10cm-wide arms In agreement with Hritcu L *et al.*[14], minor changes were made. Short-term spatial working memory is indicated by spontaneous alternation.

Morris Water Maze Test

Vorhees *et al*. (2006) evaluated hippocampal-dependent learning, such as long-term spatial memory and spatial memory, using "Morris-Water-Maze [MWM]. Round pool in Morris water labyrinth (210 cm diameter, 50 cm height). After induction, Vorhees CV *et al*. logged the day's data: path, speed, target quadrant (TSTQ) time, and concealed platform time[15].

Biochemical Analysis

Preparation of brain homogenat

Every animal was sacrificed using carbon dioxide on day 14. Brains were washed with extremely cold Isotonic Saline after removal. Brain-homogenized samples from each group of animals were used for the analysis of these vital markers. Freeze-dried brain homogenates were used for biochemical analysis.

The modifications were made in the methods and used to measure Superoxide Dismutase (SOD) activity[16], Glutathione (GSH) activity[17], nitric oxide (NO) level in brain,[18] Catalase (CAT)[19] and MDA level in brain.[20]

Total protein content[21]

Dilutions of tissue fractions were placed in 0.1 mL test tubes. The contents were mixed with 5.0 mL Lowry-C reagent and 0.8 mL 0.1M sodium hydroxide using a vortex mixer. The sample was replaced with distilled water as the reagent blank for 660 nm color analysis.

RESULTS AND DISCUSSION

Evaluation of Nanocochleates[22]

To make LDNC, Nanocochleates used the greatest nanoliposome. LDNC trapping produced nanocochleates. The study found that the particle size, zeta potential, drug loading percentage, and entrapment efficiency % were 91.2, -53.4, 85.06 ± 1.48, and 90.77 ± 1.39, respectively (data not provided due to previous publication). DSC spectra demonstrate that the formulation traps the medicine, changing endotherm energy. The in vitro study found that LDNC released 29.5% less drug than Free LD (43.85%) in the first hour. After four hours, LDNC was 63.77% and Free LD 98.03%. The levodopa showed a definite 2ϴ in XRD analysis, while the LDNC lacked this feature. This may be due to levodopa crystallinity diminishing. The surface morphology showed a tubular rod shape, suggesting NC formation. The FESEM examination of electrospray-created NCs corroborated our notion. Overall, nanocochleates were more powerful than free L-dopa and had better pharmacological and pharmacokinetic properties.

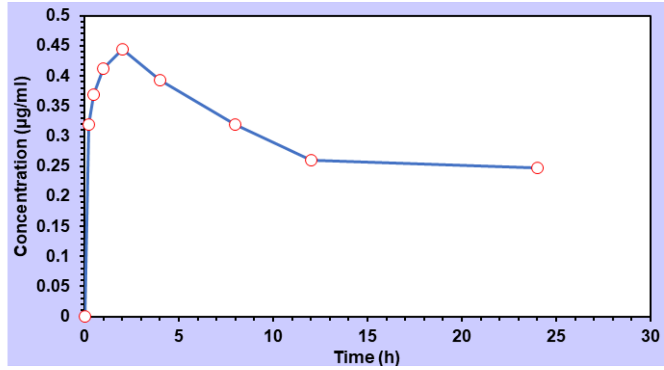

Figure 1: Pharmacokinetic study of levodopa

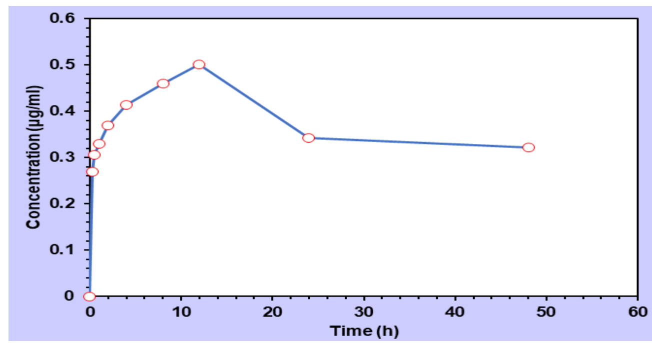

Figure 2: Pharmacokinetic study of LDNC

In-vivo Pharmacokinetics[22]

Free LD and LDNC were administered orally at 25 mg/kg. When delivered as Nanocochleates, the blood had a greater drug content. More than half as long (t1/2) as free LD (28.89 min) for LDNC. The improved LDNC formulation has a much higher Cmax than free LD. The nanocochleates formulation had worse clearance than free LD. After oral treatment, LDNC blood concentration reached 0.50 µg/mL, significantly higher than free LD (0.44 µg/mL) at Tmax 1h. A high AUC value for nanocochleates indicates that the drug was more bioavailable than free LD. Nanocochleates absorb faster and have higher medication bioavailability than LD (Figures 1 and 2).

Gradual drug release from LDNC and lymphatic absorption may boost LD's bioavailability by prolonging blood residence. A non-compartmental model examined pharmacokinetic parameters using PK Solver. All values were provided as mean ± standard deviation. Significant differences were deemed significant when the significance criterion ranged from $p < 0.05$ to $p < 0.001$.

Table 1: Number of crossing and rearing and time spent in the centre of the open field

Parameter{s}	*VC*	*6-OHDA*	*Free L-Dopa*	*LDNL*	*LNDC*
Number of crossings	21 ± 5.07	15.25* ± 6.14	18.375* ± 5.90	18.875* ± 6.08	20** ± 5.32
Number of rearings	16.75 ± 3.37	13.25* ± 3.06	16* ± 3.34	16.5*** ± 5.37	19.875** ± 3.36

The mean ± SEM (n = 6) is represented by each value. For the 6-ODHA group, there are fewer crossings and rearing; in contrast, administration of free L-Dopa, LDNL, and LDNC results in fewer crossings And-rearing (*p <0:05,** p <0:01,and*** p <0:001vs6-OHDA). T-test after a one-way ANOVA. 6-hydroxydopamine, or 6-OHDA

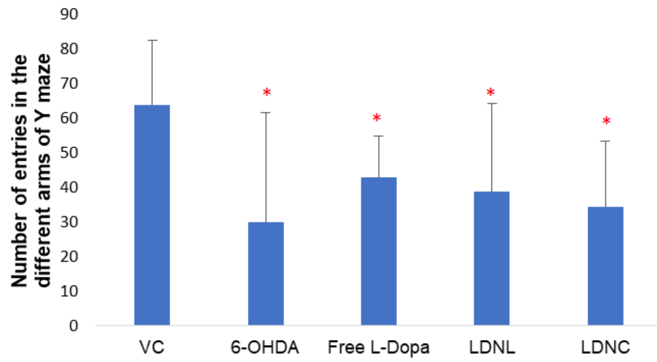

Figure 3: Effects of samples on memory in Y-Maze Test. **(**Rats were given either vehicle or free L-dopa, LDNL, or LDNC one hour prior to the test, and thirty minutes later, they received an injection of either vehicle or 6-OHDA. Total Number of arm Entries during eight-minute Y-Maze trials. Data is shown as mean ± SEM (K = 6). Significant differences were seen between the groups: ∗< 0.05 for the vehicle-treated control group compared to 6-OHDA alone group and between the samples-treated group and the 6-OHDA group).

Evaluation of formulation for Behavioural Assessment[23, 24]

Effects of LDNCin the locomotion activity in open field

The rats were given 6-OHDA to test their open-field locomotion. After crossing, rats treated with LDNC, LDNL, and free L-Dopa had significantly higher locomotor activity than those treated with 6-OHDA ($p < 0.05$) (Table 1). When comparing 6-OHDA to the control, the crossing number was lower (15.25 ± 6.14) ($p < 0.05$). Rats given LDNC exhibited more crossing and rearing activity ($p < 0.01$), while those given 6-OHDA had decreased activity ($p < 0.05$).

LDNC Effects evaluated in Y- Maze Test

6-OHDA-induced amnesia performed worse than control in the Y-maze test (Figure 3). Oral LDNC treatment (three doses to matching groups) significantly restored 6-OHDA-decreased recognition memory (Figure 3). The LDNC-treated group exhibited a higher spontaneous alternation score than the 6-OHDA-injected amnesic group. Total arm entries were similar across experimental groups under the same settings.

Effects of LDNC on in the morris water maze

Two-way ANOVA demonstrated that 6-OHDA affected escape latency, distance, average speed, and platform quadrant time in MWM. In contrast to the first session, the Vehicle-6-OHDA-treated control failed the spatial test, whereas LDNC pre-administration produced a time course and significantly reduced escape latency. A post hoc `multiple comparison test showed that 6-OHDA" treated rats spent less time on the platform than controls in the probing experiment, but LDNC pre-treatment restored this difference (Figure 4).

Biochemical evaluation[25-28]

In rats given 6-OHDA, LDNC affects brain total protein level, lipid peroxidation (MDA), GSH, NO, CAT, and SOD. 6- Rats fed only OHDA exhibit high MDA. LDNC-treated mice have normal MDA levels compared to controls. Compared to the control group, 6-OHDA alone significantly lowered Glutathione And Catalase activity. Antioxidant enzymes rise significantly in LDNC-fed rats. LDNC raised catalase activity to 0.04 mmol H_2O_2/mg protein from 0.02 in 6-OHDA. When given 6-OHDA, the LDNC group had higher glutathione levels than the control group. Although less than the therapy group, LDNC boosted glutathione and catalase. Brain effects of formulation Total protein, GSH, NO, CAT, MDA, and SOD were elevated in 6-OHDA-treated rats (Table 2).

DISCUSSION

An increasing amount of research suggests that PD symptoms include motor deficits and cognitive abnormalities that diminish quality of life. A prominent neurotoxin used in animal experiments to simulate Parkinsonism is 6-OHDA. Research suggests that 6-OHDA-induced neurotoxicity may be triggered by oxidative stress created by Hydroxyl radicals during autoxidation[5]. Extreme oxidative stress can kill neurons. Previous studies found that 6-OHDA may cause cognitive loss in animals[29] and that oxidative stress is a primary driver of memory loss[30]. 6-OHDA is suitable for simulating Parkinsonian and cognitive decline in rats.

We tested rats' cognitive ability using "Morris-Water-Maze, Y-maze, and Open Field Tests since the hippocampus is important for spatial memory[30]. These tasks are effective

Table 2: Biochemical evaluation

Groups	*SOD*	*GSH*	*NO*	*CAT*	*MDA*	*Protein*
VC	0.53 ± 0.09	0.77 ± 0.29	0.02 ± 0.001	0.05 ± 0.02	0.33 ± 0.03	0.57 ± 0.11
6-OHDA	0.24 ± 0.06	1.24 ± 0.11	0.06 ± 0.003	0.02 ± 0.001	0.59 ± 0.07	0.51 ± 0.21
LDNC	0.49 ± 0.07	0.79 ± 0.13	0.03 ± 0.002	0.04 ± 0.004	0.35 ± 0.02	0.54 ± 0.07
Free LD	0.49 ± 0.05	0.99 ± 0.07	0.05 ± 0.006	0.03 ± 0.002	0.41 ± 0.03	0.51 ± 0.07
LDNL	0.44 ± 0.08	0.81 ± 0.08	0.03 ± 0.006	0.03 ± 0.001	0.36 ± 0.05	0.52 ± 0.05

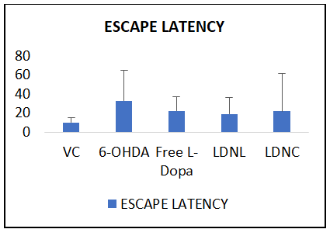

Figure 4a

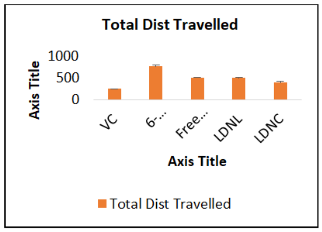

Figure 4b

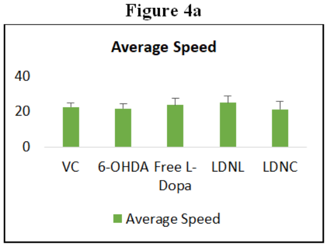

Figure 4c

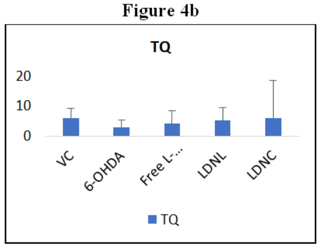

Figure 4d

Figure 4 (a), (b), (c) and (d): Impact of Levodopa and its formulations on Morris Water Maze test. Escape latencies (a) and swimming distance (b) Average speed (c) and time spent in platform Quadrant (d) in rats with 6-OHDA memory impairment during Morris Water Maze task trial sessions. Data are; shown as mean ± SEM, with n = 6 for each group

for assessing rodent spatial learning and memory[24]. Parkinson patients' brains show increased lipid peroxidation and decreased free radical scavenging enzymes.[27,28] Neuronal death from oxidative stress follows "6-OHDA-model. Thus, we quantify MDA and scavenging enzyme activity. This study found that a 6-OHDA injection dramatically increases MDA and decreases scavenging enzyme levels, suggesting that free radicals may cause cognitive impairment in PD.MDA levels drop to normal in LDNC-treated animals compared to controls. 6-OHDA alone significantly reduced glutathione and catalase, with LDNC showing a minor rebound. LNDC significantly boosts antioxidant enzyme levels in rats.

This study revealed that levodopa-loaded Nanocochleates, liposomes, and free levodopa may protect against this toxicity. By modulating antioxidant status, oral LD, LDNL, and LDNC reduced behavioral abnormalities and protected the rat's striatum from oxidative stress 6-OHDA exposure. In our study, LDNC treatment reduced 6-OHDA-induced cognitive loss and retained spatial memory better than levodopa alone, likely due to its stability and oxidation protection.

Our findings further show that the toxin caused an oxidative chain reaction in the striatum by creating ROS and reducing TRAP and GSH. Increasing SOD and GR activity may be an adaptive response to rising RS and falling GSH and TRAP levels. This surge was counterbalanced by increased SOD activity, which generated a lot of H_2O_2.[28,29]

CONCLUSION

Effective neurodegenerative disease treatment is clinically unmet. Parkinson's disease-related dementia is poorly investigated. Quality of life is greatly reduced by dementia. Current medications are not effectively treating the issue. Many studies support the assumption that oxidative stress causes disease. However, inadequate dosage, low bioavailability, restricted CNS Transport and temporary retention, and insufficient antioxidant capability to entirely reduce free radical effects may have hampered their clinical application. Nano-particle-based drug delivery methods may overcome several of the above issues. Recently, much work has been done to establish a nano-therapy-based activities administration approach. An effective antioxidant system may assist various clinical conditions because many diseases share oxidative stress as a pathophysiological process.

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