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Research Article

PRAMIPEXOLE DIHYDROCHLORIDE LOADED MPEG-PCL NANOSUSPENSION BY MODIFIED NANOPRECIPITATION: *IN VITRO* AND *IN VIVO* EVALUATION

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ABSTRACT

Objective: Nanosuspension in drug delivery is known to improve solubility, dissolution, and eventually bioavailability of the drugs in the brain. This study was carried out to show the neuroprotective effect of pramipexole dihydrochloride nanosuspension for the potential treatment in Parkinson's disease and to evaluate the effectiveness in both *in vitro* and *in vivo* models.

Methods: Nanosuspension of pramipexole dihydrochloride was prepared with methoxy polyethylene glycol-poly (caprolactone) (MPEG-PCL) and Pluronic F68 by the process of modified nanoprecipitation technique with different concentrations of MPEG-PCL. The particle size, zeta potential, scanning electron microscopy (SEM), transmission electron microscopy (TEM), and *in vitro* drug release were performed. The cell viability study was performed using SH-SY5Y cells. Further, the formulation is evaluated for its antioxidant potential against rotenone-induced neuronal damage in male Wister rats such as enzymatic, non-enzymatic antioxidants, and histopathological evaluation.

Result: The nanoformulation shows least particle size of 143 nm and maximum zeta potential value of 33.4 mv with 88.53% entrapment efficiency were observed with PMPNP 2 formulation. The SEM, TEM, and *in vitro* drug release of PMPNP 2 were showed spherical shape with controlled release when compared to other formulations. Further, the MTT assay was performed using SH-SY5Y cells which show more than 50% cell viability with 50 µl of PPMNP 2 nanoformulation. Moreover, the antioxidant potential done in rotenone-induced neuronal damage in Wister rats. The results showed elevation in the levels of enzymatic and non-enzymatic antioxidants compared with the neuronal toxic group, and the nanoformulation group showed a decrease in levels of lipid peroxidation which correlates with histopathological architecture.

Conclusion: This study concluded that our nanoformulation shows a significant advantage of neuroprotective effect than the plain formulation for the treatment of Parkinson's disease in both *in vitro* and *in vivo* studies.

Keywords: Pramipexoledihydrochloride, Methoxy polyethylene glycol-poly (caprolactone), SH-SY5Y cells, Nanoprecipitation, Parkinson's disease.

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INTRODUCTION

The Parkinson's disease is a more prevalent in person with about 65 years of age concomitant with genetic influence, toxins, oxidative stress, mitochondrial abnormalities, and alpha-synuclein aggregation in a higher rate, which is considered to be the main cause for Parkinson's disease. It is the second most prevalent neurodegenerative extrapyramidal motor disorder succeeding Alzheimer's disease. Mostly the cause is Idiopathic. Mutation in chromosome 4 and 6 is the main origin for Parkinsonism [1,2]. Dopamine is a neurotransmitter which is feasible in performing the functions of lokomotion, learning, working, memory, cognition, and emotion. These functions are performed by binding to their specific receptors which are the members of large G-protein coupled receptor. The deregulation of the dopaminergic system has a correlation with Parkinson's disease, generation of pituitary tumors, Tourette's syndrome, schizophrenia, and attention deficit hyperactive disorder [3].

Apparently, the brain needs a regular supply of nutrients to maintain homeostasis and other vital function. The blood-brain barrier (BBB) represents an effective obstacle for the delivery of neuroactive agents to the central nervous system and it makes the treatment of much brain disease is difficult to achieve. Many researches reveal limitations over oral therapy due to the restricted entry of the drug into brain due to its hydrophilicity, necessitating frequent dosing and cholinergic side effects such as severe bradycardia, nausea, dyspepsia, vomiting, and anorexia. Hence, the present aim is to formulate the nanoparticulate systems that can improve brain targeting provide sustained release, minimize side effects, and reduce dosing frequency [4,5]. The drug is dissolved, entrapped, encapsulated or attached to a nanoparticle (NP) and depending on the method of preparation NPs, nanospheres or nanocapsules can be obtained. Considering the properties of biodegradable NPs essentially grand bioavailability, control release, less toxicity and better encapsulation, they are frequently used as a vehicle in drug delivery. Biodegradable polymers such as methoxy polyethylene glycol-poly (caprolactone) (MPEG-PCL), poly lactic-co-glycolic acid, poly lactic acid, chitosan, gelatin, polycaprolactone, and poly-alkyl-cyanoacrylales are used for nanoformulations [6,7]. MPEG-PCL which possesses the idiosyncratic features such as non-toxicity, hydrophilicity, biodegradability, mechanical strength, biocompatibility, and physical inertness used as a carrier for targeted drug delivery in neurodegenerative disorder [8,9]. It possesses higher penetration capacity, absorption across the mucosal epithelia, the potential of binding with various ligand molecules and helps in the formation of stable nanocomplex [10-12]. We previously demonstrated that brain-targeted NPs can transport large amounts of BBB-impermeable agents across the BBB on systemic administration [13]. Pramipexole dihydrochloride is a well-known antiparkinsonism drug which produces toxicity as a side effect with the frequent administration in dose regimen only a minimal amount of the drug crosses the BBB. However, loading ant Parkinsonism drug to NPs and their transport to the brain in an active form and sufficient concentrations remained a challenge. In this study, we have the first developed the conditions to load primaxazole to NPs and then performed the characterization, entrapment efficiency and *in vitro* drug release. We have also demonstrated the significant neuroprotective effect of NP through upregulation of antioxidant status. Hence, this study is carried out to enhance the targeted drug delivery of Pramipexole dihydrochloride in combination with polymers for the treatment of Parkinson's disease.

METHODS

Pramipexole dihydrochloride, MPEG-PCL and Pluronic F 68 were purchased from Sigma-Aldrich, Bengaluru, India. All the other chemicals were used as an analytical grade.

Preparation of pramipexole dihydrochloride nanosuspension

The preparation of pramipexole dihydrochloride nanosuspension is carried by modified nanoprecipitation method [14,15]. In this method, phosphate buffer with pH 9.0 was used as an external medium instead of aqueous phase. Various concentrations ranging 10-50 mg of MPEG-PCL and 10 mg pramipexole dihydrochloride were accurately weighed and dissolved in 5 ml acetone. This organic solution was added slowly to pluronic F 68 (1%) in phosphate buffer (pH 9.0) solution. The organic solvent was then allowed to evaporate for 2 hrs with continuous stirring on a magnetic stirrer (Remi). The NP suspension was then centrifuged at 15,000 rpm for 30 minutes at 4°C using high-speed centrifuge (Remi). The supernatant was taken for further evaluation.

Particle size and zeta potential

The size of the prepared NPs was analyzed using Malvern apparatus. All samples were diluted with ultra-purified water, and the analysis was performed at a scattering angle of 90° and at a temperature of 25°C. The mean diameter for each sample and mean hydrodynamic diameter was generated by cumulative analysis in triplicate. The zeta potential is determined using a zeta seizer. The measurements were performed using an aqueous dip cell in an automatic mode by placing diluted samples in the capillary measurement cell and cell position is adjusted.

Scanning electron microscopy (SEM)

The surface morphology of the NP suspension was studied using SEM quanta 200 FEG scanning electron microscope (FEI Quanta FEG 200) set at 200 kV by placing an air dried NP suspension on copper electron microscopy grids, and the image was captured at desired magnification.

Transmission electron microscopy (TEM)

TEM analysis of the prepared formulations was carried out to understand the morphology of NP suspension. A drop of NPs suspension containing 0.01% of phosphotungstic acid was placed on a carbon film coated on a copper grid for TEM. TEM studies were performed at 80 kV. The copper grid was fixed into sample holder and placed in a vacuum chamber of the TEM and observed under low vacuum, and the images were recorded.

Drug content

Drug content was determined by taking 1 ml of the MPEG-PCL loaded Pramipexole dihydrochloride nanosuspension. To this formulation 1 ml of aqueous potassium dihydrogen phosphate solution (30 mM) was added and the mixture was centrifuged at 10,000 rpm at 15°C. The clear supernatant was removed and analyzed by spectrophotomertically, also drug content where calculated.

Drug entrapment efficiency

The drug loaded NPs are centrifuged at 13,000 rpm for 30 minutes and the supernatant is assayed for non-bound drug concentration by spectrophotometer.

In vitro release studies

In vitro release studies were performed using diffusion apparatus United States Pharmacopeia-II at 50 rpm [16], 10 ml of the nanoformulation was placed in dialysis membrane having molecular weight cut-off from 12,000 to 14,000 daltons. The membrane was soaked in phosphate buffer saline (PBS) for 12 hrs before using [13]. Pramipexole dihydrochloride formulation in dialysis membrane was placed in the bowl containing 100 ml of PBS pH 7.4 at fixed time intervals, 1 ml of the aliquot was withdrawn and fresh PBS pH 7.4 was replaced to maintain constant volume.

In vitro studies

Analysis of cell viability

To evaluate the activity of pramipexole dihydrochloride nano-suspension protection against Parkinson's disease were MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay was performed as an *in vitro* model [17] (Kura *et al.* 2014). The MTT assay where performed using SH-SY5Y cells lines, the cells are plated separately in 96 well plates at a concentration of 1×10^5 cells/well. After 24 hrs, cells were washed twice with 100 µl of serum-free medium and starved for an hour at 37°C. After starvation, cells were treated with the test material for 24 hrs. At the end of the treatment period, the medium was aspirated and serum free medium containing MTT (0.5 mg/ml) was added and incubated for 4 hrs at 37°C in a CO_2 incubator. The MTT containing medium was then discarded and the cells were washed with PBS (200 µl). The crystals were then dissolved by adding 100 µl of dimethyl sulfoxide and this was mixed properly by pipetting up and down.

In vivo studies

Adult male Wistar rats, approximately weighing 150-200 g were housed in a temperature-controlled room and maintained on 12:12-hrs light/dark cycles, with free access to food and water. The animals were handled according to the principles of laboratory care framed by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Chennai, The experimental protocol was reviewed and approved by the Institutional Animal Ethics Committee (IAEC) has approved for animal studies and the approval no is XV/VELS/PCOL/10/2000/CPCSEA/IAEC/30.10.13.

Dosing and treatment

Animal was divided into 4 groups and each group containing 6 animals. Group A treated as control rats received saline (2 ml/kg body weight by per oral route) for 10 days. Group B treated rats received rotenone (2.5 mg/kg body weight) dissolved in saline and administered intraperitoneally for 10 days [18].Group C rats received pramipexole dihydrochloride nano-suspension (PMPNP 2) (drug equivalent to 0.8 mg/ml/day/kg body weight) was administered by oral gavage once daily 30 minutes before rotenone (2.5 mg/kg body weight) dissolved in saline for 10 days. Group D rats received pramipexole dihydrochloride pure drug (1 mg/kg body weight/day) was administered by oral gavage once daily 30 minutes before rotenone (2.5 mg/kg body weight) dissolved in saline for 10 days. After the treatment, all the animals were anesthetized and decapitated. Brain tissues were immediately excised and rinsed in ice-cold physiological saline and used for various evaluations.

Biochemical studies

Enzymatic antioxidant estimation

Estimation of superoxide dismutase (SOD)

Briefly, 2.8 ml of reactive mixture (xanthine 0.3 mM, ethylenediaminetetraacetic acid [EDTA] 0.67 mM, 150 µM nitrotetrazolium blue chloride, sodium carbonate 0.4 M, bovine albumin 30 mg/30 ml) is added to 0.1 ml tissue homogenate and 50 µl xanthine oxidase (10 µl in 2 M ammonium sulfate), incubated at 25°C for 20 minutes and mixed with 0.1 ml 8 M copper chloride. The color reaction was measured at 560 nm [19].

Estimation of catalase (CAT)

A total of 100 µl of tissue homogenate was placed in an ice bath for 30 minutes and then for another 30 minutes at room temperature. A total of 10 µl triton-X 100 was added to each tube. In a cuvette containing 200 µl phosphate buffer and 50 µl of tissue homogenate, 250 µl of 0.066 M H_2O_2 was added (in phosphate buffer) and a decrease in optical density was measured at 240 nm for 60 seconds. The molar extinction coefficient of 43.6 M/cm was used to determine CAT activity. One unit of activity is equal to the moles of H_2O_2 degraded/minutes per mg protein [20].

Estimation of glutathione (GSH) perodiase (GPx)

A reaction mixture consisting of 700 µl phosphate buffer (0.05 M containing 0.01 mM EDTA, PH 7.0), 100 µl 0.01 M GSH (reduced form), 100 µl 1 mM nicotinamide adenine dinucleotide phosphate (NADPH), and 100 µl GSH reductase (GR) (0.24 units) was used. Tissue homogenate (50 µl) was added to the reaction mixture and incubated at 37°C for 10 minutes. Then, 50 µl of 12 mM cumene hydroperoxide was added to the reaction mixture, and the absorbance was measured at

340 nm for 180 seconds on a spectrophotometer. The molar extinction coefficient of 6.22×10^3 cm/L was used to determine the activity of GPx. One unit of enzyme activity is equivalent to 1 mM of NADPH oxidized/minutes/mg protein [21].

Non-enzymatic antioxidant estimation

Estimation of GSH

The tissue homogenate was centrifuged at 1700 rpm for 10 minutes. The enzymatic reaction was started by adding 100 µl of clear supernatant in a spectrophotometric cuvette containing 800 µl of 03 mM reduced nicotinamide adenine dinucleotide phosphate, 100 µl of 6 mM 5,5-dithiobis-2-nitrobenzoic acid and 10 µl of 50 units/ml GR (all these reagents were freshly prepared in a phosphate buffer at pH 7.5). The absorbance was measured over a period of 4 minutes at 412 nm at 30°C. The GSH level was determined by comparing the rate of change of absorbance of the test solution with that of standard GSH [22].

Lipid peroxidation (LPO)

To determine LPO, 10% homogenate was prepared using 0.01 mol/L Tris-HCl buffer, pH 7.4 and was used immediately. Briefly, 0.1 ml of homogenate was added to the test tube containing 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid solution, pH 3.5, and 1.5 ml of 0.8% thiobarbituric acid (TBA) solution. The mixture was diluted to 4.0 ml with distilled water and heated at 95°C for 60 minutes. After cooling on ice, the samples were extracted with 4.0 ml of mixture of n-butanol and pyridine (15:1, v/v). After centrifugation at 3000 rpm for 10 minutes, the organic phase was collected, and the absorbance was measured at 532 nm. The concentration of 2-TBA was determined using the extinction coefficient of 1.56×10^5 L/m cm. The results were expressed as nanomoles of malondialdehyde (MDA) released per milligram protein [23].

Histopathology studies

For histopathology studies, the brain tissue fixed in 10% formalin for 4 days and dehydrated in 50, 70, 95 and 100% ethanol, 20 minutes each time, and then submerged in xylene twice, 10 minutes each time. Then, sections were taken and stained with hematoxylin-eosin. Digital microphotographs were taken at ×400 magnification.

Statistical analysis

All data were expressed as mean±standard to the number of experiments. The statistical significance was evaluated by one-way analysis of variance using SPSS version 13.0 (SPSS), and the individual comparison was obtained. A value of $p<0.05$ was considered to indicate a significant difference between groups.

RESULTS AND DISCUSSION

Preparation of pramipexole dihydrochloride nanosuspension

The preparation of pramipexole dihydrochloride nanosuspension is carried out by modified nanoprecipitation method and the formula for the pramipexole nanosuspension is given in Table 1.

Particle size and zeta potential

The particle sizes were shown in Table 2 which describes the increase in polymer concentration having an impact on particle size. The pramipexole dihydrochloride loaded MPEG-PCL nanoformulations of 1:2 shows 143 nm in the table may be chosen as better particle size because another ratio of 1:1 having less polymer so that more drug may not be encapsulated. On the other hand, 1:3, 1:4, 1:5 are having more amount of polymer with high zeta potential, hence the 1:2 ratio with 143 nm particle size with 33.4 mv zeta is chosen due to the higher value of zeta potential implies more stable. The size and stability may be compromised to achieve a better bioavailability.

SEM

The SEM analysis was performed for the polymeric nanosuspension after selecting appropriate field and magnification. The SEM photographs are shown in Figs. 1 and 2. It shows the morphological characters of PMPNP 2 nanoformulation. It is conferred that the particles are in nano sizes, and the particle shape was found to be spherical shaped in appearance.

TEM

TEM image shows internal composition such as morphology, crystallization, stress or even magnetic domains. Fig. 3 shows that the polymeric NPs of PMPNP 2 were smooth, spherical, and uniform.

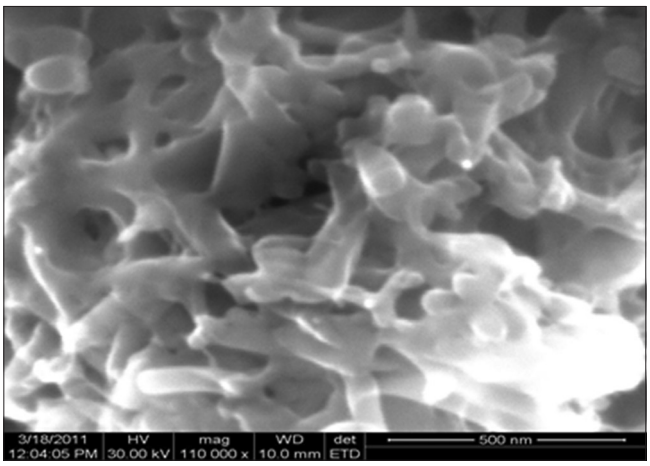

Fig. 1: Scanning electron microscopy photos of pramipexole dihydrochloride loaded methoxy polyethylene glycol-poly (caprolactone) nanosuspension (PMPNP 2)

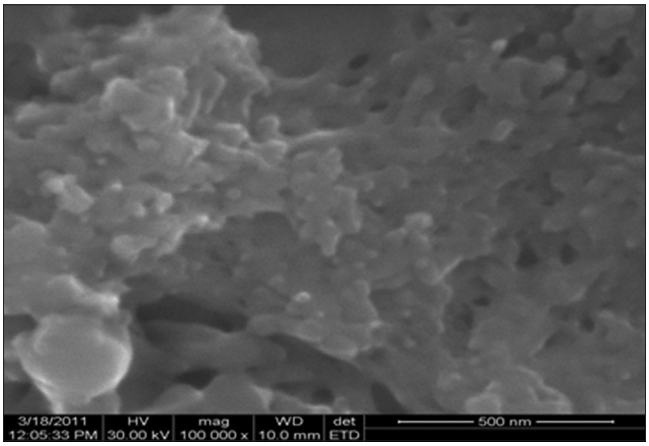

Fig. 2: Scanning electron microscopy photos of pramipexole dihydrochloride loaded methoxy polyethylene glycol-poly (caprolactone) nanosuspension (PMPNP 2)

Table 1: Formulation of pramipexoledihydrochloride MPEG-PCL nanosuspension

Ingredients	PMPNP 1	PMPNP 2	PMPNP 3	PMPNP 4	PMPNP 5
Pramipexole dihydrochloride (mg)	10	10	10	10	10
MPEG-PCL (mg)	10	20	30	40	50
Pluronic F 68 (%)	1	1	1	1	1
Acetone (ml)	5	5	5	5	5

MPEG-PCL: Methoxy polyethylene glycol-poly (caprolactone)

Drug content and entrapment efficiency

The drug content of the prepared nanoformulations were determined and the results shows 0.59 mg/ml with 88.53% entrapment efficiency in PMPNP 2 with higher drug content having more entrapment efficiency which was shown in Table 3 since other formulation shows low drugs content when compare to PMPNP 2 with varying entrapment efficiency. Thus, based on result PMPNP 2 may be best formulation among the remaining nanosuspension.

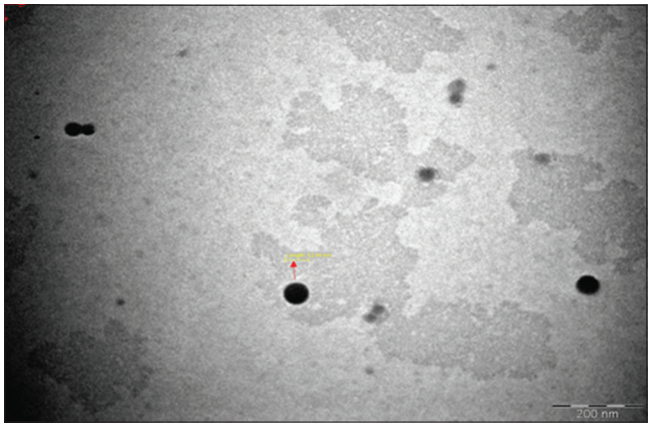

Fig. 3: TEM photos of pramipexole dihydrochloride loaded methoxy polyethylene glycol-poly (caprolactone) nanosuspension (PMPNP 2)

Table 2: Particle size and zeta potential analysis of optimized pramipexole dihydrochloride loaded MPEG-PCL A nanosuspension

Formulation	Ratio	Particle size (nm)*	Zeta potential (mv)*
PMPNP 1	1:1	140±2.5	33.2±2.7
PMPNP 2	1:2	143±1.6	33.4±1.1
PMPNP 3	1:3	155±2.2	34.8±1.5
PMPNP 4	1:4	182±1.3	33.4±1.4
PMPNP 5	1:5	220±4.2	32.2±3.3

*Values indicated in the results of triplicate trials±SEM. MPEG-PCL: Methoxy polyethylene glycol-poly (caprolactone)

Table 3: Drug content and entrapment on efficiency of pramipexole dihydrochloride loaded MPEG-PCL nanosuspension

Formulation	Ratio	Average drug content mg/ml*	Average entrapment efficiency (%)*
PMPNP 1	1:1	0.45±0.04	57.28±2.14
PMPNP 2	1:2	0.59±0.10	88.53±1.42
PMPNP 3	1:3	0.47±0.06	68.21±1.72
PMPNP 4	1:4	0.43±0.09	69.13±2.04
PMPNP 5	1:5	0.35±0.14	80.27±1.24

*Values indicated in the results of triplicate trials±SEM. MPEG-PCL: Methoxy polyethylene glycol-poly (caprolactone)

***In vitro* release studies**

Table 4 shows *in vitro* drug release of all formulations; in that, PMPNP 1 shows the highest drug release but was rejected as the particle size was very high PMPNP 3, PMPNP 4, and PMPNP 5 were not selected despite low particle size because they act as release retardants due to a high concentration of polymer. Hence, PMPNP 2 is selected as the best formulation due to its optimum drug release 96.53 in 24 hrs which is an controlled release than 16 hrs in PMPNP 1 and 76.77%, 68.98%, 58.33% in PMPNP 3, PMPNP 4, PMPNP 5 formulation even after 24 hrs the drug was no released, hence based on results we obtained PMPNP 2 may be best formulation. The selected formulation was taken for further cell viability studies and *in vivo* studies.

Cell viability studies

Cell viability studies were performed by MTT assay. It depends on mitochondrial dehydrogenases for MTT conversion and these enzymes are also inhibited by MPP which is shown in Fig. 5 with decreased in the cells which is in the monolayer formation. The pramipexole dihydrochloride nanosuspension on MPP was assayed at various concentrations from 1 to 50 µg/ml (Fig. 6a-e) its shows dose-dependent recovery of cells were seen at concentrations of 5, 10 and 50 µg/ml. The recovery of cells at 50 µg/ml of pramipexole dihydrochloride recovery of cells in Fig. 7e was nearly equal to control which is in Fig. 4 and also compared with pure drug of Fig. 7a-d shows same as nanosuspension. The plain nanosuspension with MPP caused a significant decrease in cell viability, and NPs suspension showed dose-dependent protection.

Fig. 8 shows the graph of percentage of cell viability in SH-SY5Y cells lines where Parkinsonism was induced by MPP. The result shows more than 50% of viable cells in nanoformulation with 5 µl treated against MPP induced cell which is similar when compared to the pure drug of 5 µg/ml treated cells. Hence, the polymeric nanoformulation may be more effective for the treatment of Parkinsonism diseases.

Enzymatic antioxidants estimation

Table 5 represents that the activities of enzymatic antioxidants (SOD, CAT and GPx) were significantly ($p<0.05$) lower in rat brain tissue of rotenone-induced (Group B) rats as compared to that of control (Group A) rats. This is due to the damage of mitochondria by rotenone which leads to the reduction in antioxidant enzyme levels. Enzymatic antioxidant is required to protect the cells from apoptosis. The treatment of rat brain tissue (Group C) with nanoformulations significantly ($p<0.05$) reversed all these rotenone -induced alterations in the activities of endogenous antioxidants (SOD, CAT and GPx) to a near normal status. The rats receiving pure drug alone (Group D) show significant change when compared with rotenone-induced, indicating

Table 4: *In vitro* release studies of pramipexole dihydrochloride loaded MPEG-PCL nanosuspension

S. No.	Time (hrs)	% Cumulative drug release*				
		PMPNP 1	PMPNP 2	PMPNP 3	PMPNP 4	PMPNP 5
1	0	0	0	0	0	0
2	1	7.22±0.2	7.12±0.3	5.99±0.4	6.32±1.2	5.87±1.0
3	2	21.85±1.4	11.91±1.2	14.90±1.4	12.62±1.3	10.40±1.3
4	4	35.67±1.6	25.43±1.6	37.65±2.4	24.68±1.1	19.39±1.1
5	6	58.52±1.8	38.66±1.5	37.65±2.4	36.52±1.2	25.26±1.2
6	8	74.06±1.3	43.88±0.5	37.65±2.4	45.67±1.4	30.29±1.2
7	12	92.56±0.6	62.13±1.2	53.74±1.6	52.17±1.3	41.38±1.3
8	16	100±0.4	69.84±1.0	58.84±1.2	60.16±1.1	48.32±1.4
9	20	-	87.90±1.2	65.38±1.4	64.45±1.3	52.43±1.3
10	24	-	96.53±1.3	76.77±1.1	68.98±1.2	58.33±1.4

*Values indicated in the results of triplicate trials±SEM. MPEG-PCL: Methoxy polyethylene glycol-poly (caprolactone)

Table 5: The enzymatic antioxidants evaluation of rat brain in experimental animals

Groups	Parameters		
	(Units/mg protein)		
	SOD	CAT	GPx
Group A (Control)	1.28±0.051	2.74±0.055	1.54±0.70
Group B	0.37±0.066	0.97±0.071	0.59±0.63
Group C	0.98±0.033	2.64±0.045	1.48±0.032
Group D	1.30±0.042	2.71±0.054	1.52±0.046

±SD mean p<0.05. SOD: Superoxide dismutase, CAT: Catalase, GPx: Glutathione perodiase

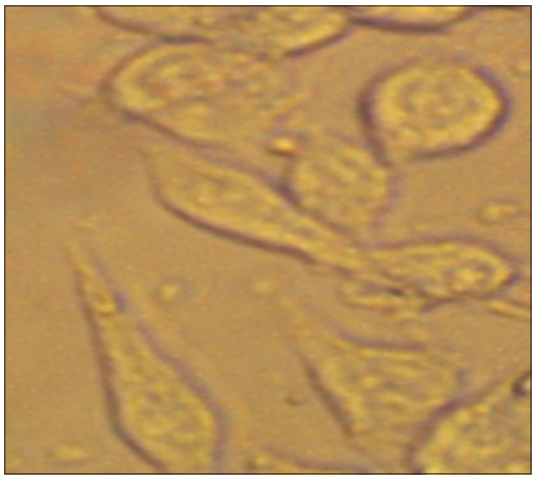

Fig. 4: The plates of MTT assay of control

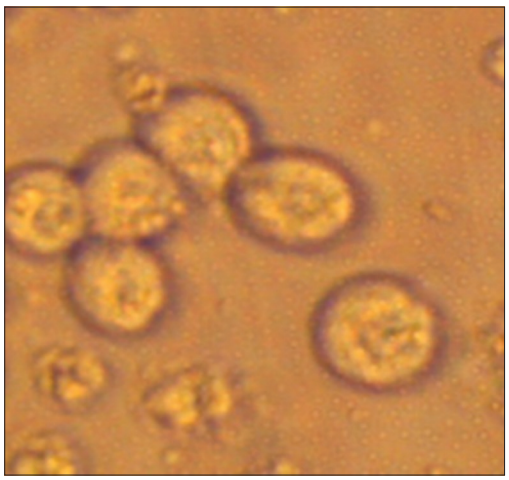

Fig. 5: The plates of MTT assay of MPP

the protection of cell from apoptotic cell death which restore of enzymatic antioxidants potential with pramipexole dihydrochloride nano-suspension.

GSH and LPO estimation

Table 6 shows the level of GSH in rat brain. The levels of GSH were significantly (p<0.05) lower in rat brain of rotenone-induced rats (Group B) when compared to control rats (Group A). The GSH level is reversed by the nanoformulations + rotenone cotreated rats (Group C) which show the alteration in the levels when compared to the rotenone-induced rats (Group B). The rats receiving pure drug alone (Group D) show significant change when compared with rotenone-induced. Table 6 shows the level of MDA in rat brain. The

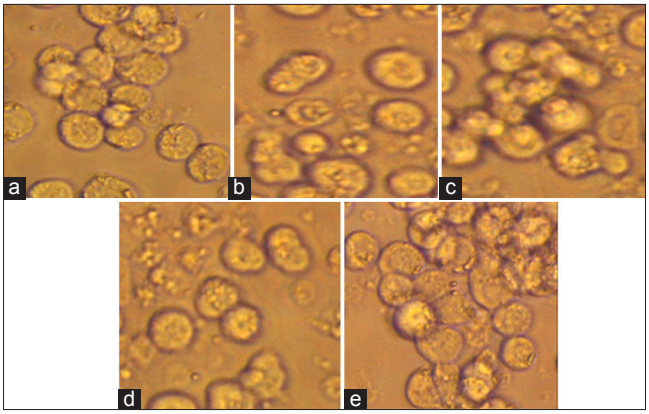

Fig. 6: The plates of MTT assay of MPP and pramipexole dihydrochloride pure drug in different concentration. (a) MPP+pure drug 1 µg/ml. (b) MPP+pure drug 2 µg/ml. (c) MPP+pure drug 5 µg/ml. (d) MPP+pure drug 10 µg/ml. (e) MPP+pure drug 50 µg/ml

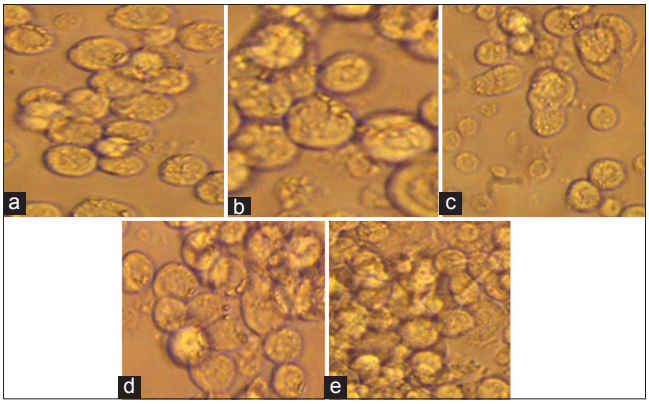

Fig. 7: The plates of MTT assay of MPP and pramipexole dihydrochloride nanosuspension loaded methoxy polyethylene glycol-poly (caprolactone) in different concentration. (a) MPP+PMPNP 2 1 µl. (b) MPP+PMPNP 2 2 µl. (c) MPP+PMPNP 2 5 µl. (d) MPP+PMPNP 2 10 µl. (e) MPP+PMPNP 2 50 µl

levels of MDA were significantly (p<0.05) higher in rat brain tissue of rotenone-induced rats (Group B) when compared to control rat brain tissue (Group A). The increase levels of MDA lead to higher the rate of peroxides may lose membrane strength of cells further leads to cell death. The MDA level is reversed by the nanoformulations + rotenone co-treated rat brain tissue (Group C and Group E) which shows the alteration in the levels when compared to the rotenone-induced rat brain tissue (Group B). The rats receiving pure drug alone (Group D) does not show significant change when compared with rotenone -induced, indicating the increase in membrane strength shows the recovery of cell by the pramipexole dihydrochloride nano-suspension.

Histopathology evaluation

Histopathology photographs of rat brain in an experimental animal are in Fig. 9 The group A (Fig 9a) as control, where the report observed in rotenone-induced Group B (Fig 9b) rat shows cell shrinkage along with degeneration of nucleus and accumulation of more nucleus in one cell leads to cell damage. This has been reversed by pramipexole dihydrochloride nanosuspension treated Group C (Fig 9c) when compared to Group B (Fig 9b). Whereas the Group D (Fig 9d) shows the same level of protection, which supports the nanoformulation treatment reveals the rotenone-induced degeneration of neural cells which further supports the pramipexole dihydrochloride nanosuspension is a more effective in the treatment of Parkinsonism diseases.

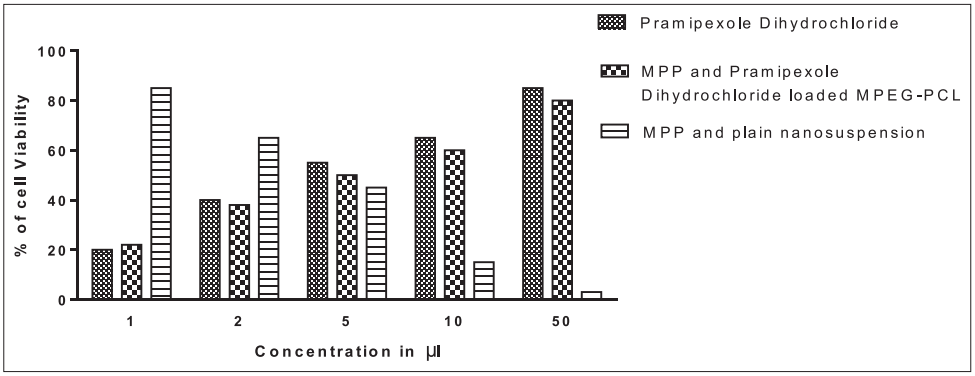

Fig. 8: The graph of MTT assay of MPP with pure pramipexole dihydrochloride drug, pramipexole dihydrochloride loaded methoxy polyethylene glycol-poly (caprolactone) nanosuspension and plain nanosuspension in different concentration

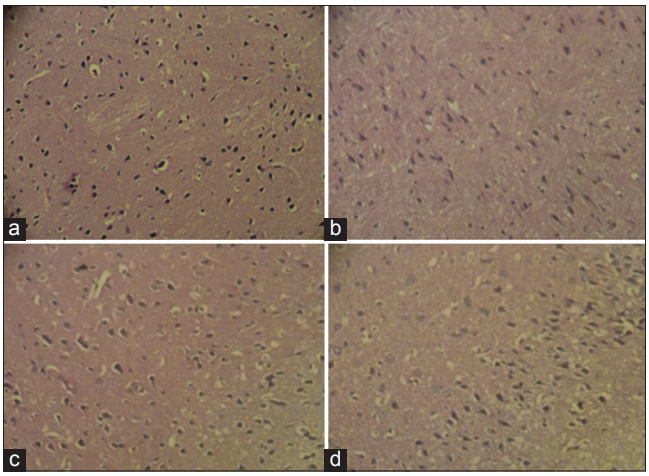

Fig. 9: The plates of histopathological evaluation of rat brain in experimental animal. (a) Group A - Control (normal saline). (b) Group B - rotenone (2.5 mg/kg). (c) Group C - PMPNP 2 (drug equivalent to 0.8 mg/ml/day/kg body weight)+rotenone (2.5 mg/kg). (d) Group D - pramipexole dihydrochloride (1 mg/kg)+rotenone (2.5 mg/kg)

Table 6: The GSH and LPO levels of rat brain in experimental animals

Groups	Parameters	
	(µmole/mg protein)	
	LPO	GSH
Group A (control)	0.46±0.012	0.52±0.050
Group B	0.18±0.015	0.11±0.041
Group C	0.40±0.004	0.42±0.035
Group D	0.43±0.006	0.52±0.027

±SD mean p<0.05. GSH: Glutathione, LPO: Lipid peroxidation

CONCLUSION

We have prepared and characterized the primaxazole dihydrochloride nanosuspension and showed potential neuroprotective effects in both *in vitro* and *in vivo*. The administration of nanosuspension significantly rescued the neuronal perturbations compared to the nonformulated pure drug which lends a hand in the enhancement of primaxazole dihydrochloride nanosuspension to cross the BBB for the treatment in Parkinson's disease.

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