

Attenuated levels of phospholipids in the striatum of rats infused with rotenone causing hemiparkinsonism as detected by simple dye-lipid complex



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ABSTRACT

Parkinson's disease (PD), a progressive neurodegeneration, is characterized by loss of dopaminergic neurons in the substantia nigra (SN) and loss of motor co-ordination. Impaired metabolism of major lipids such as phospholipids which play regulatory roles in cellular functions and signaling has been implicated in the pathology of PD. We aim to investigate the striatal phospholipids (PLs) in hemiparkinsonism infused by rotenone in rats. As there are no cost-effective modes of PL, we have utilized dye-lipid complex technique for the first time in PD models for screening and also for semi-quantifying (individually) the levels of the deregulated PL in brain samples. Rats were divided into 2 groups: i. control and ii. ROT-infused which received intracranial injection of Rotenone (6 $\mu\text{g}/\mu\text{l}$; flow rate 0.2 $\mu\text{l}/\text{min}$). At the end of experimental period of 14 days, the striatum was dissected out for the analyses of PLs. Dye-based detection of PL and two-dimensional thin-layer chromatographic analyses of PL were performed. Detection of dye-PL complex was possible for phosphatidyl choline (PC), phosphatidyl inositol (PI), and spingomyelin (SM) (but not for phosphatidyl ethanolamine-PE) using dyes viz victoria blue B, toluidine blue and ammonium ferrothiocyanate, respectively. Two-dimensional analyses of phospholipids confirmed the dye-PL complex and depicted significant reduction ($p < 0.05$) on semi-quantitative assessment, in the striatum of control and hemiparkinsonic rats. We suggest a low level of PLs esp of PI in striatum of rats using a simple dye-detection that was validated by HR-LCMS. The finding implies that a critical role is being played by these PLs (PC, PI and SM) mainly PI ($p < 0.001$), in rotenone infused hemiparkinsonism, thus deserving wider but simpler investigations to detect and identify their role in parkinsonism.

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Abbreviations: PD, Parkinson's disease; AD, Alzheimer's disease; DA, Dopaminergic neuron; SNpc, Substantia nigra pars compacta; ROT, Rotenone; DMSO, Dimethyl sulfoxide; PLs, Phospholipids; PC, Phosphatidyl choline; PE, Phosphatidyl ethanolamine; PI, Phosphoinositide; SM, Spingomyelin; NM, Neuromelanin; TLC, Thin layer chromatography; VBB, Victoria blue-B dye; TB, Toluidine blue dye; AFTC, Ammonium ferrothiocyanate; ANSA, 1 Amino-2 naphthol-4 sulfonic acid; HCl, Hydrochloric acid; Na.EDTA, Sodium Ethylenediaminetetraacetic acid; NaCl, Sodium chloride; TBAHS, tetrabutyl ammonium hydrogen sulphate; HRLCMS, High resolution liquid chromatography–mass spectrometry; ESI, Electrospray ionization; MS, Mass spectrometry; MRM, Multiple reaction monitor; TEM, Transmission electron microscopy; PI(3,5)P₂, Phosphatidylinositol 3,5-bisphosphate; PtdIns(4,5)P₂, Phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4,5)P₃, Phosphatidylinositol 3,4,5-trisphosphate; PtdIns5P, Phosphatidylinositol 5-phosphate.

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1. Introduction

Parkinson's disease (PD) is characterized by selective degeneration of A9 dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc), which causes a severe depletion in striatal dopamine (Ehringer and Hornykiewicz, 1960; Marsden, 1982; Hornykiewicz and Kish, 1986). PD is a unique human disease that is characterized by symptoms such as akinesia, bradykinesia, depression and muscle tremor (Savitt et al., 2006). The formations of fibrillar cytoplasmic inclusions are known as Lewy bodies (Betarbet et al., 2000), which contain ubiquitin and α -synuclein. α -synuclein is the main component of lewy bodies, the hallmark of parkinsonism and the defective gene-product interaction involving α -synuclein can aggravate the condition of neurodegeneration, due to the fact that α -synuclein can exert a strong interaction with phospholipids leading to changes in membrane dynamics and vesicular transports (Jao et al., 2004; Cooper et al., 2006;

Abd-Elhadi et al., 2015; Feany and Bender, 2000). Phospholipids (glycerophospholipids and sphingolipids) that are important building blocks of cell membranes, provide an optimal environment for protein interactions, trafficking and function (Wenk and De Camilli, 2004).

Phospholipids (PL) actively participate in signal transduction in response to both external and internal stimuli, and providing precursors for signaling processes and macromolecular synthesis. The essential functions include provision of membranes required for protein synthesis and export, cholesterol homeostasis, and triacylglycerol storage and secretion (Lagace and Ridgway, 2013). Phospholipid metabolism in the endoplasmic reticulum with perturbations in lipid storage or secretion and stress responses, ultimately contributes to obesity or diabetes, atherosclerosis and neurological disorders (Lagace and Ridgway, 2013). Alzheimer's disease (AD) associated proteins were found to influence lipid metabolic pathways in the brain. Phosphatidyl choline (PC), spingomyelin (SM), phosphatidyl inositol (PI), and phosphatidyl ethanolamine (PE) are important components of nervous tissue and lipid rafts (Devane et al., 1992; Pécheur et al., 2002). Thus, they are critical for neural communication and neurotransmission (Choi and Chun, 2013) both of which are highly impaired in neurodegenerative conditions like parkinsonism (Chan-Palay, 1990).

The cause and mechanisms of pathological complications of parkinsonism in the majority of cases remain largely unknown. An association has been reported between PD and exposure to mitochondrial complex-I toxins, including environmental pesticides paraquat, maneb, and rotenone (Tanner et al., 2011). Rotenone is commonly used natural pesticide extracted from the roots of tropical plants, such as *Derris elliptica*, can able to freely cross the blood brain barrier, plasma membrane, mitochondrial membranes and radiolabeled (3H) dihydrorotenone binds to striatal sections from rodent brains with a Kd of ≈ 55 nM (Higgins and Greenamyre, 1996) and induces PD-like symptoms, include dopaminergic neurodegeneration and the occurrence of cytoplasmic inclusions similar to Lewy bodies (Betarbet et al., 2000).

We aimed at evaluating the pathological involvement of phospholipid in PD models using simple TLC and Dye complex method (further validated by sensitive HR-LCMS) as the detection of PL is, generally, highly challenging that involve costly procedures, with the anticipation that these phosphometabolites could function both as markers and targets understanding parkinsonic features and also for building strategies of therapies.

2. Materials and methods

2.1. Materials

Rotenone and phosphoinositide were purchased from Sigma-Aldrich, St. Louis, MO, USA. All chemicals used for the study were of analytical grade. TLC plates were purchased from DC-Fertig folien alugram-Xtra SIL G/UV 254.

2.2. Animals

Adult male *Sprague-Dawley* rats (250–300 g) were used in the present study. The animals were maintained under standard conditions of 12 h light/dark cycles (lights on at 7:00 AM), 22 ± 1 °C temperature and $60 \pm 5\%$ of relative humidity. They were provided food and domestic quality tap water available *ad libitum*. The experimental protocols met the National CPCSEA Guidelines on "Proper Care and Use of Animals in Laboratory Research" (Indian National Science Academy, New Delhi) and appropriate care has been taken

to minimize the sufferings of the animals, and to limit the number of animals used in the experiments.

2.3. Experimental design

Rats were distributed randomly into 2 groups, with 6 rats in each group: Control group, ROT-infused group unilateral; (6 $\mu\text{g}/\mu\text{l}/\text{kg}$). At the end of experimental period of 14 days, the rats were sacrificed and dissection of the striatum was carried out for further experiments.

2.4. Stereotaxic surgery

Rats were anesthetized with chloral hydrate (450 mg/kg, i.p.). The animal was placed in the flat skull position on a stereotaxic frame (Instrument & Chemicals Pvt Ltd., Ambala city, India) with incisor bar fixed at 3.5 mm below the interaural line. ROT was dissolved in 1:1 DMSO and polyethylene glycol (6 $\mu\text{g}/\mu\text{l}/\text{kg}$) and then it was infused into the right SNpc at a flow rate of 0.2 $\mu\text{l}/\text{min}$, using a micro infusion pump which consists of a Hamilton micro syringe (Reno, Nevada, USA). The craniotomy was sealed with bone wax and the overlying skin incision was closed. For the following infusion, 5 min were given before retracting the probe for complete diffusion of the drug. The stereotaxic coordinates were: Lateral = 0.20 cm; Antero-posterior = 0.53 cm; and Dorso-ventral = 0.75 cm from the Bregma point (Paxinos and Watson, 1998). Proper post-operative care was provided till the animals recovered completely. The rats were left in a temperature-controlled chamber until they were recovered from anesthesia, then they were returned to their home cages. Histopathological analysis was done in striatum to detect changes following ROT-infusion (Madathil et al., 2013). The animals were sacrificed on day 14 for the following histological and biochemical experiments.

2.5. Brain tissue collection

The rats were sacrificed by cervical decapitation in the morning to avoid diurnal variations of the endogenous amines, enzymes and other antioxidant molecules. The regions of striatum from the brain of rats were dissected out, separated and weighed. The brain tissues were excised and examined under microscope ensuring the sections.

2.6. Histopathological analysis

Histopathological analyses was used to confirm the neurodegeneration in ROT-infused rats. The striatum of control and ROT-infused rats was dissected and fixed in 10% neutral buffered formalin before embedding in paraffin. Five micrometer sections were cut, serially dehydrated in an ethanol gradient and stained with hematoxylin and eosin (H and E) before being examined under a light microscope.

2.7. Transmission Electron Microscopy (TEM)

A small amount of striatum tissue was cut into small pieces of 1 mm \times 1 mm \times 1 mm, fixed in 2.5% glutaraldehyde for 24 h, post-fixed in osmic acid for 1 h, dehydrated with graded acetone, and finally embedded with epoxy resin Epon 812. Using the ultramicrotome, the tissue was cut into 50 nm ultrathin slices, put on the copper grid and stored at 4 °C. In a clean petri dish, a drop of 3% uranyl acetate-alcohol dye liquor (pH-3.5) was added to the ultrathin slices for 30 min, rinsed in H₂O for 10 min three times, and water was drained. Using the same method, the slices were dyed by 6% lead citrate dye liquor (pH-12) for 5 min, water was

drained and the sections were placed at room temperature for the ultra-structure observation under TEM (Hitachi H-600 TEM).

2.8. Extraction and purification of phospholipids

The lipids were isolated by the method of Folch et al. (1957). A known weight of striatum in rat brain tissue is homogenized with chloroform/methanol (2:1) mixed with 10 ml of solvent mixture. The homogenate was filtered through Whatman No. 42 filter paper into a separating funnel. The filtrate was mixed with 0.2 ml of normal saline and the mixer kept overnight undisturbed, the lower phase containing the lipid was drained off into pre weighed beakers. The upper phase was re-extracted with more solvent mixer and the extracts were pooled and evaporated to dryness at room temperature. The lipid extract was dissolved in 3.0 ml of the solvent mixer and aliquots were taken for purification.

2.9. Dye-PL assessment complex

2.9.1. Victoria blue-B dye

To each aliquot of the supernatant, 0.5 ml of Victoria blue-B (VBB) solution in ethylene glycol/glycerol (1/1, v/v) mixture (13.5 mg/100 ml) was added with chloroform to make the final volume 2.8 ml. Test tubes were shaken vigorously. Then 1.0 ml of ethylene glycol was added to each tube and the biphasic system was vigorously mixed again. After 3–5 min of centrifugation, 500 g per minute, the upper phase was removed by aspiration and the lower chloroform phase was transferred with a Pasteur pipette into an 1 cm beam cuvette for excitation and emission spectra measurement and were read in the spectrofluorimeter. Blanks containing all additions except phospholipids.

2.9.2. Toluidine blue dye

To each aliquot of the supernatant, 0.5 ml of Toluidine blue (TB) solution was added with chloroform to make the final volume 2.8 ml. Test tubes were shaken vigorously. Then 1.0 ml of ethylene glycol was added to each tube and the biphasic system was vigorously mixed again. After 3–5 min of centrifugation, 500 g per minute, the upper phase was removed by aspiration and the lower chloroform phase was transferred with a Pasteur pipette into an 1 cm beam cuvette for excitation and emission spectra measurement and were read in the spectrofluorimeter.

2.9.3. Ammonium ferrothiocyanate dye

To each aliquot of the supernatant, 2 ml of chloroform was added followed by the addition of 1 ml ammonium ferrothiocyanate. Test tubes were shaken vigorously and the biphasic system was vigorously mixed again. After 3–5 min of centrifugation, 500 g per minute, the upper phase was removed by aspiration and the lower chloroform phase was transferred with a Pasteur pipette into an 1 cm beam cuvette for excitation and emission spectra measurement and were read in the spectrofluorimeter.

2.10. Two-dimensional chromatography for phospholipids

Plates were air-dried overnight and activated prior to use by heating for at least 1 h at 120 °C. Lipids were dissolved in chloroform 10 µl samples and were applied as elliptical spots to the origin of TLC plate. The plate was first developed with a solvent system consists of chloroform–methanol–28% ammonia (65:25:5, v/v/v). After drying, the plate was developed in the second dimension with a solvent system consists of chloroform–acetone–methanol–glacial acetic acid–water (50:20:10:10:5, v/v/v/v/v). Plates were air-dried and stained with iodine vapor prior to charring of spots by heating for 30 min at 190 °C.

2.11. Extraction of PLs from TLC spots

Silica gel was scrapped from the tested areas of TLC plates into the tubes, each of which contained 200 µl of the hexane/isopropanol/1.5 N NH₄OH (24/16/1, v/v) extraction mixture. In the supernatant, the semi-quantitative determination of phospholipid content lecithin, cephalin and sphingomyelin was carried out.

2.12. Estimation of inorganic phosphorous as an indirect semi-quantification of phospholipids

The phosphorous content of the extracted from tissues were estimated by the method of Fiske and Subbarow's method 1925, after digesting the lipid extract with perchloric acid. 0.1 ml of dried lipid extract was dissolved in 1 ml of conc. sulphuric acid and 72% perchloric acid (9:1 v/v) and kept in a sand bath until the solution became colorless. After cooling, the solution was made up to 5 ml with distilled water. To all the tubes 9.6 ml of 0.26% ammonium molybdate and 0.4 ml of 3% ANSA were added and the mixture was kept in a boiling water bath for 6 min. The blue color developed was read at 710 nm using a Shimadzu UV spectrophotometer. Phospholipids content is expressed in µg/mg of tissue.

2.13. Extraction of phosphoinositide from striatum

A known weight of striatum was homogenized by the method of Pettitt (2010) with a nitrogen-cooled pulverizer and 1 ml ice cold methanolic HCl (0.25 M) until fully disrupted. The homogenate was transferred into a 15 ml centrifuge tube using a pipette and the homogenizer were rinsed with 2 ml of methanolic 0.25 M HCl/chloroform (1:1) and combined with homogenate. Again the homogenizer was rinsed with 3 ml of chloroform and pooled with homogenate. Vortexed vigorously for 30 s and sonicated in an ice-cooled sonicating bath for 10 min. The homogenate was phase separated by the addition of 1.5 ml 0.25 M HCl, 2 mM Na₂EDTA, 5 mM tetrabutyl ammonium hydrogen sulphate (TBAHS; 1.7 mg/ml), and 0.9% (155 mM; 9 mg/ml) NaCl. The mixture was vortexed for 30 s, which sonicated in an ice-cooled sonicating bath for 5 min and kept on ice for 10 min after which complete phase separation was done by centrifugation (1100 rpm, 5 min). The lower phase was transferred into a tube containing 1.5 ml of 100 mM Na₂EDTA (pH-6.0) and 1.5 ml 1 mM TBAHS in methanol. This neutralizes the acid extract, the remaining upper phase and interfacial material were re-extracted again as above and their lower phases were pooled. The combined lower phases were vortexed vigorously and the phase separation was completed by centrifugation (1100 rpm, 5 min). The upper phase was discarded and the interfacial material was dried under evaporator. Then, the tube walls were carefully rinsed with 500 µl chloroform/methanol/water (5:5:1) dried and resuspended in 30 µl chloroform/methanol/water (49:49:2). The contents were transferred into silanized 1.8 ml auto sampler vial using a glass syringe and 30 µl of chloroform/methanol/water (5:5:1) was added in silanized vial to give a total volume of ≈60 µl. The samples were analyzed quickly to minimize any losses on standing (possibility for slow acid hydrolysis).

2.14. HR-LCMS analysis

Semi-quantification of PI was based on the separation was performed on an Agilent series 1290 infinity UHPLC system coupled to an ESI, 6550 ifunnel single quadrupole mass spectrometer equipped with a turbo-assisted ionspray (ESI) ionization source (Agilent Technologies, USA) in positive mode. Analyst MPP software was used for instrument control, data acquisition and data analyses. The sample vials were maintained at

5 °C in the thermostatic autosampler. Chromatographic separation was obtained using gradient elution on a Silica (3 m, 1.0 × 150 mm) column at 15 °C at a flow rate of 80 µl/min. Separation was performed using a binary gradient composed of mobile phase solvent A; chloroform/dichloromethane/methanol/water (45:45:9.5:0.5) containing 15 mM ethylamine, Solvent B; acetonitrile/chloroform/methanol/water (30:30:32:8) containing 15 mM ethylamine, Solvent C; 60 mM piperidine in methanol and needle wash solvent; chloroform/methanol/water (49:49:2). The injection volume was 0.5 µl and the eluent flow was diverted to waste for 2 min after sample injection and again after 13 min. MS detection was carried out between 1 and 15 min of the chromatography, total run time of the analyses including recycling was 35 min. The mass spectrometer was operated in positive mode.

2.15. Statistical analysis

Statistical analyses were performed using the Prism 6.00 Software (Graph Pad Software for Windows, La Jolla, CA, USA). Summarized data was expressed as mean ± S.E.M. Differences between groups were statistically analyzed by one-way ANOVA followed by Dunnett's post-hoc test depending on the homogeneity of variance test. In all cases, $p < 0.05$ was considered statistically significant.

3. Results

3.1. Histopathological H&E staining of striatum

Rotenone 6 µg dose of rotenone resulted in significant cell shrinkage/morphological alteration in the striatum after 14 days of treatment. The striatum of control animals (Fig. 1A) showed normal histology of round to oval shaped neurons with lesser intensity of staining. Striatum of ROT-infused rats showed reveal abnormal striatal histology with hyperstained irregularly shaped neurons along with vacuolation of neurophil as well as microcyst of neurophils and swelling of neurons (Fig. 1B).

3.2. Ultra-structures changes in striatum under TEM

To analyze nuclei, mitochondria, synaptic vesicles and lipid droplets as well as ultrastructural injuries or inflammation. TEM analysis was performed. In the striatum of control rats, healthy neurons were observed with normal nuclei, dispersed chromatin and mitochondria with double membranes and clear cristae along with synaptic vesicles with a spherical shape and lipid droplets (Fig. 2A).

ROT-infused striatum had exhibited the shrunken nuclei in the neurons and condensed chromatin. The mitochondria were markedly swollen and vacuolated and the cristae were either lessened or disappeared with alterations in morphology. Reduced number of synaptic vesicles with inflammation of lipid droplets were also observed on exposure to ROT (Fig. 2B).

3.3. Chromatographic separation of phospholipids from control and ROT-infused brain tissue of rats using two-dimensional TLC

Two-dimensional chromatographic staining of standards such as phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol, sphingomyelin, control and ROT-infused was represented in Fig. 3A along with R_f values as obtained two-dimensional TLC (Table 1). As shown in Fig. 3B and C decreased levels of PC and PE were found in the striatum of a ROT-infused group when compared with those of control ($p < 0.01$). The levels of PI were remarkably decreased in ROT-infused group when compared with those of control ($p < 0.001$) (Fig. 3E), There was a reduction in the level of SM also ($p < 0.01$) in the striatum on ROT-infusion (Fig. 3D).

3.4. Dye-PL complex

We have analyzed the individual PLs in the control and in the ROT-infused neurodegenerated striatum of rats using dye methods. VBB showed excitation and emission spectra at 375/390 nm and the presence of PC 375/393 nm in the dye complex (Fig. 4A and D) but they did not form complex with PI and SM. Lecithin could form complexes with dye VBB as also found by Eryomin and Poznyakov (1989) and gets specifically partitioned into the chloroform phase of the chloroform/ethylene glycol biphasic solvent system. While the dye exhibited 375/390 nm excitation and emission characteristic of the free dye in water or alcohol solutions, complexes of PC-VBB exhibit similar spectra of excitation and emission (Fig. 4D). In the solvent system used in the study, the upper ethylene glycol-based phase selectively removes the free dye from the lower phospholipid-containing chloroform phase. Water, hydrophilic substances, particles of silica gel, partitioned or floated into the upper phase. The red inorganic compound ammonium ferrothiocyanate (AFTC) is insoluble in chloroform (Stewart, 1980). At room temperature, when a solution of chloroform containing SM is mixed well with AFTC, a colored complex ($\lambda_{395/400}$ nm) is formed which partitions in the chloroform phase. In our study, AFTC showed spectra of excitation and emission at 392/396 nm and the presence of SM in the dye complex (Fig. 4C and F) but they did not form complex with PI and PC. Toluidine blue (TB) is basic thiazine

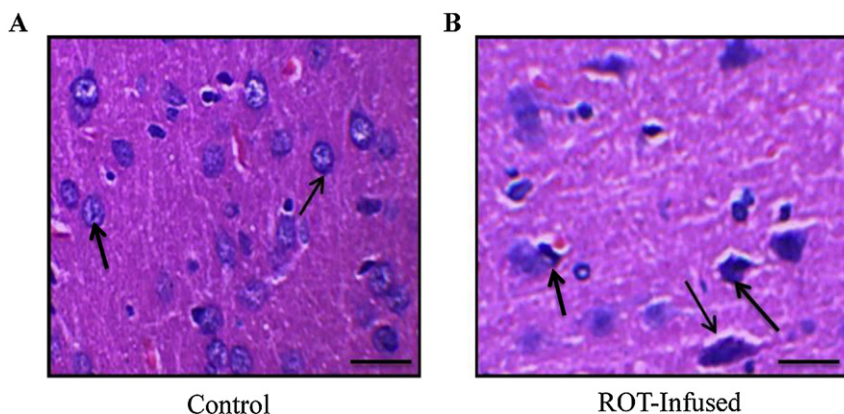


Fig. 1. Hematoxylin and eosin (H and E) staining of striatum (A) Sections from control animals show normal histology in the striatum with round to oval shaped cells (B) ROT-infused rat sections reveal abnormal striatal histology with hyperstained irregularly shaped cells along with vacuolation of neurophil as well as microcyst of neurophils and swelling of neurons. Sections stained with hematoxylin and eosin ×400. (indicated by arrows).

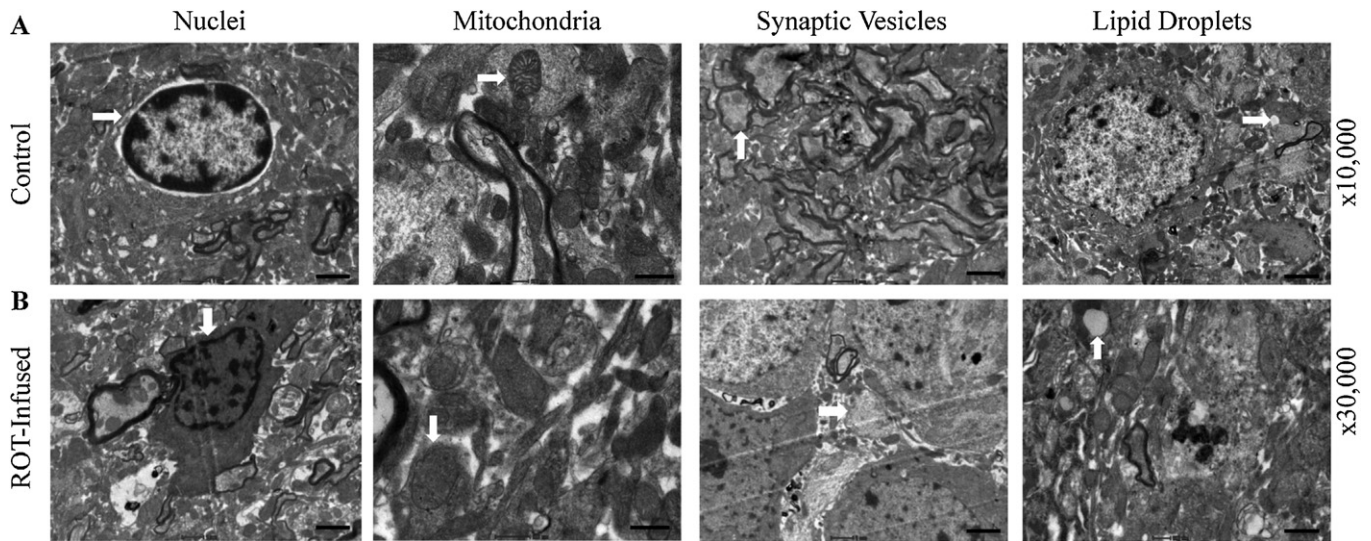


Fig. 2. Transmission electron microscopy micrographs of the striatum ($n = 6$) representing the ultrastructure of nuclei, mitochondria, Synaptic vesicles and Lipid droplets. (A) Striatal sections of control depict intact structures. (white arrows, $\times 10,000$ magnification) and (B) ROT-infused rats depict the shrunken nuclei and condensed chromatin. The mitochondria were noticeably swollen and vacuolated cristae and also decreased number of synaptic vesicles along with inflammation of lipid droplets (white arrows, $\times 30,000$ magnification) as compared to control.

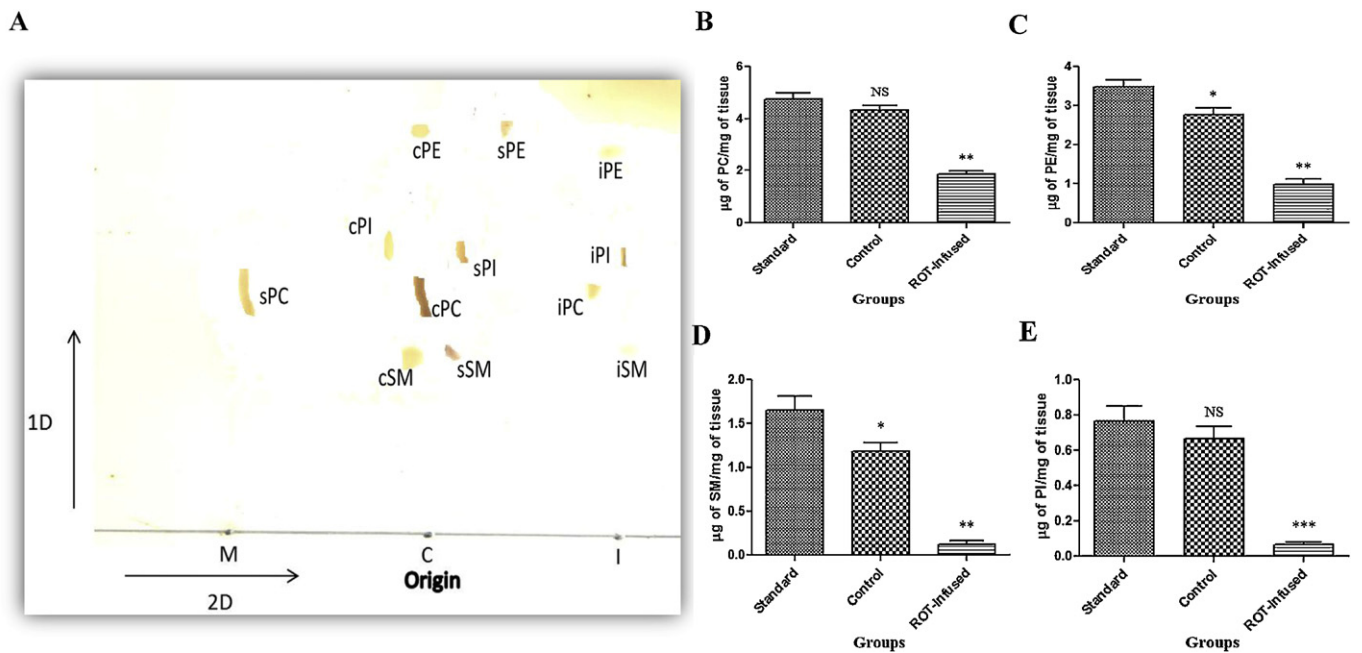


Fig. 3. Separation of phospholipids in Two-dimensional TLC and semi-quantification by inorganic phosphorous estimation in control and ROT-infused rats. [c,i,s control, infused (rotenone) and standard respectively]. Values were expressed as mean \pm SEM ($n = 6$). Each samples were repeated thrice, *** $p < 0.001$, ** $p < 0.01$ (ROT-infused Vs Control) * $p < 0.05$ and NS-Non significant (Control Vs Standard).

Table 1

R_f values as obtained two-dimensional TLC of individual phospholipids of standard, control and samples hemiparkinsonism.

Standards	R_f values		
	Standard	Control	ROT-Infused
Phosphatidyl choline	0.41	0.40	0.40
Phosphatidyl ethanolamine	0.84	0.84	0.83
Phosphatidyl inositol	0.61	0.60	0.60
Spingomyelin	0.37	0.36	0.36

metachromatic dye that could partly soluble in water and alcohol but insoluble in chloroform (Gandolfo et al., 2006). The dye showed spectra of excitation and emission at 375/400 nm (Fig. 4B and E) and bind with PI, but they did not form complex with PC and SM.

3.5. Determination of phosphoinositide using HR-LCMS

Single quadrupole mass spectrometry was used to quantify PI control and ROT-infused rats. The single Q1 ion pairs were monitored for each sample. Multiple reaction monitoring (MRM) studies were carried out to investigate the fragmentation of the precursor ions into their product ions and establish the collision energy required, so as to provide optimum intensity for each product. This

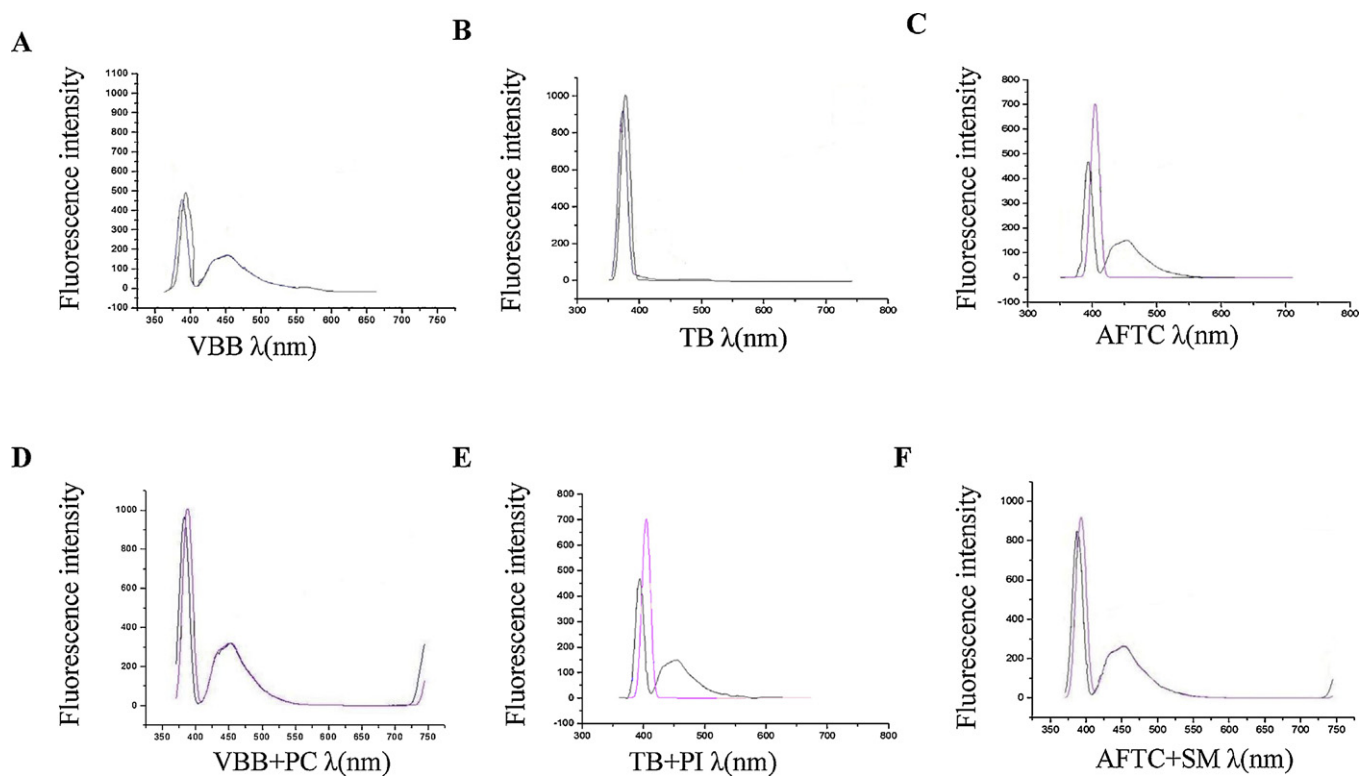


Fig. 4. Fluorescence Spectroscopic of Dye-PLs and their complex with PC, PI and SM. Fluorescence excitation and emission spectra ($\lambda_{ex}/\lambda_{em}$) of dyes and their complexes with PL: VBB-375/390 nm; VBB + PC-375/393 nm; TB-370/395 nm; TB + PI-375/400 nm; AFTC-395/400 nm; AFTC + SM-392/396 nm.

was done by setting up sequences with methods containing various collision energies for the graphs of these MRM studies (Fig. 5). The reported values were based on signal height or signal area of the single ion chromatogram at the appropriate retention time (Sommer et al., 2006). As shown in Fig. 5, dramatically decreased levels of PI(3,5)P₂ were found in the striatum of ROT-infused group when compared with those of control ($p < 0.001$).

4. Discussion

Phospholipids, the most abundant and amphipathic lipids of membranes are of great importance for cellular functions. Their dynamic adaptation to specific requirements is a crucial feature of lipid membranes in mammalian tissues. Among the phospholipids forming the bilayer, the major classes are the glycerophospholipids, phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl serine (PS) and, phosphatidyl inositol (PI) as well as sphingomyelin (SM), a sphingolipid, based on their fatty acid chains. Eukaryotic cells are able to fine-tune the mixture of these lipids according to their biological task (Wenk and De Camilli, 2004).

PC is the most abundant phospholipid in most eukaryotic cells. Choline enters cells through specific membrane transporters, is phosphorylated by Choline kinase. In case of (SM), the choline head group is transferred from PC to ceramide, in the Golgi. SM is an important component of myelin sheaths, found in the large quantities in brain and nervous tissues and very small amount of other tissues. It does not contain glycerol. In place of glycerol, it contains an 18 carbon unsaturated amino alcohol is known as sphingosine (sphingol). Another PL, PE is structurally identical with lecithin and precursor for the synthesis of anandamide (N-arachidonylethanolamine), the ligand for cannabinoid receptors in the brain (Devane et al., 1992) with the exception that the base ethanolamine replaces the choline. PE plays an important role in contractile ring disassembly at the cleavage furrow during cytokinesis of mammalian cells (Emoto et al., 1996; Emoto

and Umeda, 2000). PE also regulates the fusion of mitotic Golgi membranes (Pécheur et al., 2002). Phosphoinositides comprise a family of 8 minor membrane lipids which play important roles in many signal transducing pathways in the cell. Signaling through various phosphoinositides has been shown to mediate cell growth and proliferation, apoptosis, cytoskeletal changes, insulin action and vesicle trafficking to modulation of ion channels and survival (Eryomin and Poznyakov, 1989).

The signaling roles of phosphoinositides were first discovered by Hokin and Hokin (1955). In neurons and neurosecretory cells, whose main function is to communicate through the release of neurotransmitter, most of the work has focused on the role played by phosphatidyl inositol (4,5) bisphosphate in controlling the mechanism underpinning neurotransmitter release through the fusion of secretory vesicles with the plasmalemma. The emerging evidence supports a multi-faceted regulation of neuroexocytosis by 3-phosphorylated phosphoinositides (Wen et al., 2011).

As PLs are known to form lipophilic complexes with certain dyes and salts, we have adopted that approach to investigate the status of PL profile in the striatum of rat brain on induction of hemiparkinsonism. The application and sensitivity of the methods depend on specificity stoichiometry of the phospholipid-dye interaction, by the free and phospholipid-bound dye distribution between hydrophobic and hydrophilic phases, and by the chromophore (Eryomin and Poznyakov, 1989).

The possible reasons for variation in the levels of observed in the striatum of parkinsonic rats could probably be the decreased levels of the precursors of PLs, choline and ethanolamine, or an increase in PL turnover (Prasad et al., 1998) on ROT-infusion. This reduction of phospholipids within the brain will impact on the cell viability by a reduction in membrane stability and permeability, which would ultimately lead to cellular death (Wright et al., 2004). As the cell membrane integrity plays an important role in maintaining normal cellular function, the observed alterations in the ROT-infused group are anticipated to affect the membrane integrity resulting in the

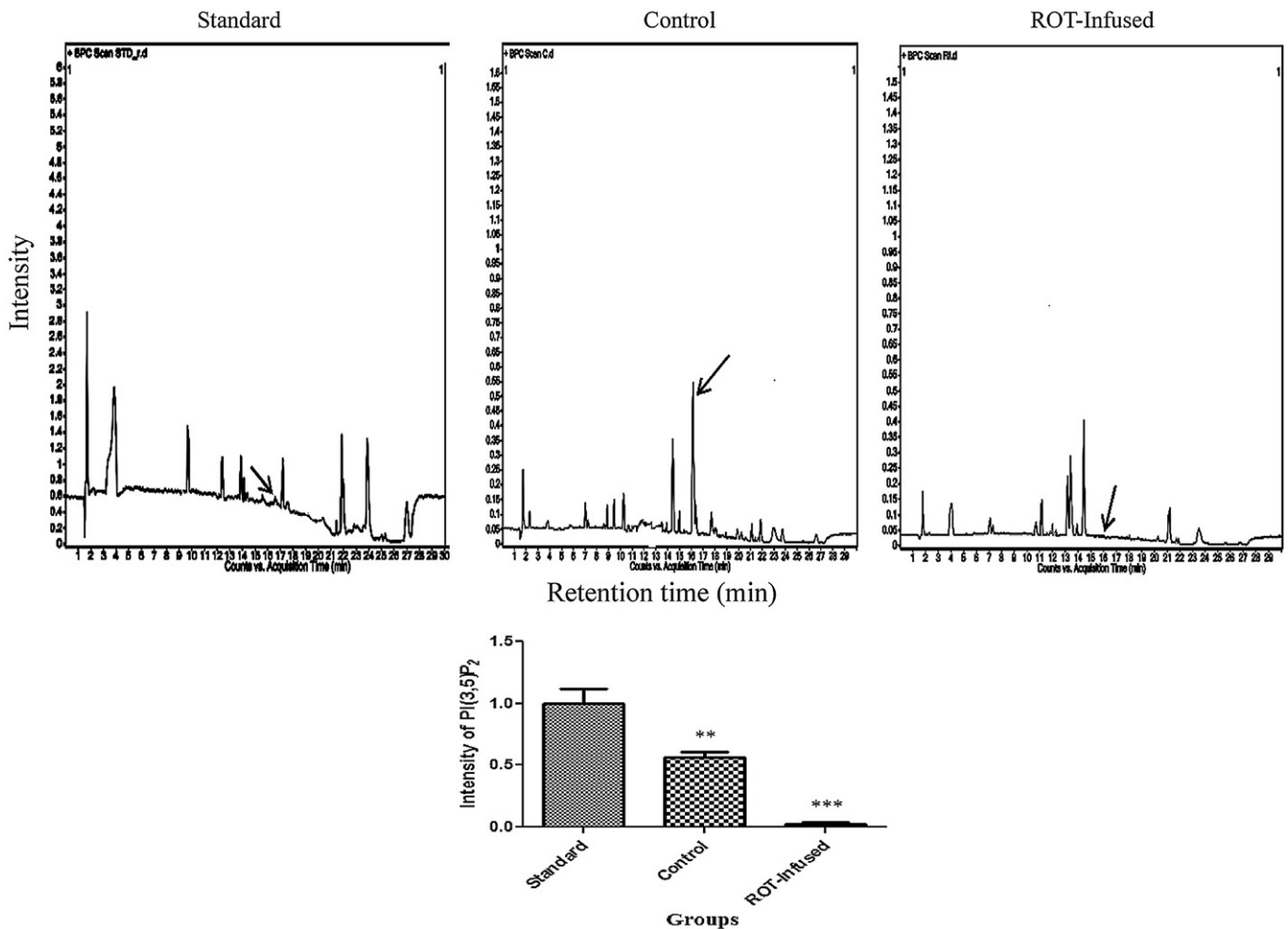


Fig. 5. HR-LCMS separation of phosphoinositides – PI(3,5)P₂ in control and ROT-infused rats. Values were expressed as mean ± SEM (n = 6). Each samples were repeated thrice, ***p < 0.001 (ROT-infused Vs Control) **p < 0.01 (Control Vs Standard).

loss of ion homeostasis, changes in activities of membrane bound enzymes, ion channels (Farooqui et al., 1997). The reduction of PC in the ROT-infused group might lead to hardening of the cell walls, thus affecting influx and efflux of cellular components and cause degeneration as PC is reported to govern the permeability (Heron et al., 1980; Higgins and Flicker, 2000).

Nigrostriatal degeneration following ROT administration both systemically (Sherer et al., 2003) and into the SNpc (Madathil et al., 2013) have been reported. The associated histological changes in brain on ROT-infusion suggest the occurrence of neuronal damage, which are manifested as pathological alteration in neuronal cell size, abnormal striatal histology with hyperstained irregularly shaped cells along with vacuolation of neutrophil, microcyst of neurophils and swelling of neurons (Fig. 1). The ultrastructure analysis by TEM of ROT-infused striatum had confirmed the neurodegeneration as depicted by shrunken nuclei in the neurons and condensed chromatin. The irregular multi vesicular bodies, dense filamentous material, reduced synaptic vesicles and inflammation of lipid droplets were found suggesting that neurodegeneration in the striatum was apparent after ROT-infusion (Fig. 2).

In the ROT-infused group, the level of PI of striatum was significantly decreased than any other PLs under investigation. Hence, the semi-quantification of PI was further confirmed by performing

HR-LCMS (Fig. 5). Interestingly, our data have shown that ROT-infusion could result in reduction of PI(3,5)P₂ in the striatum. This pioneering observation (Fig. 5) could be causing alterations in the vesicular traffic as PI are said to control the budding and fusion process between membranes and hence are critical regulators of vesicular trafficking (Balla et al., 2009) and regulate ions channels, pumps and transporters as well as both endocytic and exocytic processes. Mutation in synaptojanin 1, a polyphosphoinositides 5-phosphatase promotes the hydrolysis of both PtdIns(4,5)P₂ (Sakisaka et al., 1997) and PtdIns(3,4,5)P₃ (Woscholski et al., 1997) Synaptojanin 1 is the most abundant isoform expressed in the brain and is enriched at the level of synapses. Genetic ablation of synaptojanin 1 in mice is postnatal lethal, largely attributed to severe neurological defects (Cremona et al., 1999). Importantly, some of the neurodegenerative phenotypes caused by reductions in PI(3,5)P₂ levels could stem from increased glutamate release and excitotoxicity (Osborne et al., 2008; Tsuruta et al., 2009). The roles of PI(3,5)P₂ and PtdIns5P remain poorly understood, although they appear to be important for endocytic traffic (Ikononov et al., 2003; Sbrissa et al., 2002). Based on the existing knowledge, it could be presumed that the reduction in the levels of PI on ROT-infused hemiparkinsonism may affect vesicular traffic and also the PI mediated signaling that might affect dopaminergic neurotransmission in hemiparkinsonism.

5. Conclusion

The study concludes that ROT can alter the levels of PL in the striatum of rats with a drastic variation in the levels of PI which can be considered as useful indices aiding the detection of hemiparkinsonism and also proposes for the first time an easy-to-detect dye-PL complex method in the samples of parkinsonism, which, however do need further fine-tuning.

Conflict of interest

The authors declare no conflict of interest.

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