Original Article

# Alteration of Mitochondrial Function in Oxidative Stress in Parkinsonian Neurodegeneration: A Cross-Sectional Study

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## Abstract

**Context:** Appropriate mitochondrial function and oxidative balance are critical to neuronal survival. Accumulation of reactive oxygen species leads to oxidative stress that can cause free radical damage to biomolecules of the cell components and the molecules in the cellular milieu that eventually lead to a variety of chronic diseases including neurodegenerative disorders. Mitochondrial dysfunction initiates neuronal apoptosis thereby leading to neurodegenerative diseases including Parkinson's disease (PD). **Aim:** To evaluate oxidative stress vis-a-vis mitochondrial function (Cytochrome C oxidase activity) in PD patients, Parkinson plus syndrome (PPS) patients in comparison with healthy controls (HCs). **Settings and Design:** Cross-sectional Study **Methods:** We assessed oxidative stress by chemiluminescence using luminol, and cytochrome c oxidase activity (CCO) by CCO kit using spectrophotometry in PD patients ($n = 80$), PPS patients ($n = 40$), and HCs ($n = 40$). **Statistical Analysis:** Data were presented as number (%) or mean ± SD/median as approximate. Quantitative baseline variables were compared among the groups using one-way ANOVA and qualitative variables were compared using Chi-square test. The difference in median was compared using Kruskal–Wallis test followed by Post-hoc Bonferronni correction. **Results:** Compared to HCs (Median 7.53 ± 15.58 RLU/sec/cell), ROS level in PD (14.13 ± 29.5), and PPS (17.43 ± 15.91) patients was significantly higher ($P = 0.0029$: HC vs, PD & $P = 0.0500$: HC vs. PPS). Also, ROS in PD patients (14.13 ± 29.5) was higher that PPS patients (17. 43 ± 15.91) but the difference was not statistically significant ($P = 0.84$). The CCO activity was found to be diminished in PD (Median: 0.025 ± 0.013 units/ml) and PPS patients (0.027 ± 0.008) in comparison to HCs (0.117 ± 0.049). **Conclusion:** Mitochondrial dysfunction and oxidative stress is associated with PD and PPS and may play an important role in etiopathogenesis. Though the cause–effect conundrum has not been comprehensively probed but addressing oxidative stress and mitochondrial damage may serve as an adjunctive therapy for PD and PPS. Iron metabolism as reflected in the red cell indices may aid in differentiating PD from PPS.

**Keywords:** Cytochrome C oxidase, mitochondrial dysfunction, neurodegeneration, oxidative stress, parkinsonism, reactive oxygen species

## Introduction

Reactive oxygen species (ROS) are indispensable in cellular life.[1] They are like a necessary evil; essential in driving many biochemical transformations while extremely detrimental to cells even in slightest excess.[2] A precise balance of the quantity of ROS is necessary in and around the cells to maintain homeostasis. While decreased ROS level causes damage to many signal transduction pathways important for cell proliferation and maintenance of immune system, the ROS elevation (which is a marker of the oxidative stress) causes cellular injury and severe mitochondrial damage leading to cell death by apoptosis.[3] Oxidative stress is a condition caused by over-accumulation of ROS which causes oxidative damage to cellular biomolecules (lipids, proteins, DNA), eventually leading to many chronic diseases such as atherosclerosis, diabetics, rheumatoid arthritis, cardiovascular diseases, chronic inflammation, stroke, other major neurodegenerative diseases and even cancer.

Brain is metabolically most active organ of the body with neurons consuming 16 times more energy than muscle cells.[4] Also, the central nervous system (CNS) is comparatively deficient in antioxidants[5] making the CNS neurons relatively more susceptible to oxidative damage. Since neurons are terminally differentiated cells, oxidative damage to the tissues may be irreversible and cumulative cellular injury by elevated oxidative stress may lead to neurodegeneration, a typical example of which is Parkinson's disease (PD). Mitochondrial (Mt) dysfunction, excitotoxicity, and apoptosis have been reported to have a causative role in various other neurodegenerative diseases such as Alzheimer's disease (AD) and multiple sclerosis (MS). Literature provides substantial evidence that oxidative stress is handcuffed to the promulgation of neuronal injury in neurodegenerative

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disorders including PD and is mediated through mitochondrial dysfunction.[6,7] In support of this view, elevated oxidative stress has been reported in experimental mouse models of PD expressing α-synuclein impelled by mitochondrial dysfunction mediated through 1-methyl 4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP).[8] Relevant to PD, dopamine metabolism produces many oxidative by-products which have a tendency to oxidize α-synuclein,[9] thereby augmenting PD pathogenesis.

A variety of cellular parameters have been used as indicators of mitochondrial function; the redox state of mitochondrial cytochromes; cellular ATP levels; mitochondrial membrane potential ($\Delta\psi m$)[10]; and oxygen consumption.[11] Cytochrome C oxidase (CCO) activity is arguably the most informative of these parameters,[12,13] since this measurement allows a direct and specific assessment of the functioning of the electron transport chain (ETC), the cornerstone of oxidative phosphorylation and cellular metabolism.

In the light of these arguments, it forms a strong rationale to evaluate the oxidative status and examine the mitochondrial function in PD patients. Such an approach is important to get meaningful insights into the etiopathogenesis of PD and devise antioxidant therapies aimed at ameliorating mitochondrial dysfunction. Within this premise, the present study evaluated oxidative stress quantitatively and mitochondrial function by CCO activity in PD patients. We report that PD is associated with elevated oxidative stress and significant decline in mitochondrial function. The findings suggest that antioxidant therapies may help in preventing and/or delaying the onset of this disorder.

## Subjects

Patients diagnosed with PD attending the institute's Movement Disorder Clinic were enrolled in this study. This study was approved by the "Institute Ethics Committee." Vide Letter No: IEST/T-397/30.09.2011 Dated: 20th Oct 2011. Inclusion Criteria were: (i) age > 40 years and (ii) all stages of the disease. Exclusion criteria were: (i) patients with significant dementia (according to DSM-IV), (ii) depression/psychiatric illness (according to DSM-IV), (iii) any secondary cause of Parkinsonism, (iv) past history of stroke, (v) significant head injury, and (vi) antipsychotic medications. Controls comprised of two groups: Group II [Parkinson plus syndrome (PPS)], the recruitment of this control group was made from the patients attending the Movement Disorder Clinic. This group comprised of patients with (i) age > 40 years, (ii) PPS according to consensus criteria for MSA[14] and NINDS criteria for PSP,[15] (iii) patients with features not typical of PD. The recruitment for Group III [healthy controls (HC)] was made from age and socioeconomic status matched population. Inclusion criteria were absence of any neurological disorder, no history of alcohol consumption or psychotropic medication. Exclusion criteria were any past history of stroke, significant brain disease, uncontrolled hypertension, diabetes and smoking.

**Sample collection**: 4 ml of blood was collected from the participants by hospital phlebotomist. 400 µl was put in a heparin vial for ROS study, 600 µl in an EDTA vial for evaluating peripheral blood indices and 3 ml in a citrate vial for CCO activity estimation.

## Methods

**ROS estimation:** ROS was measured by luminol method using heparinized blood. The blood samples were kept on ice immediately after collection. The sample was processed within half an hour of collection. 400 µl of blood was taken in a Tarson vial (Product Code: 850010) and 10 µl of 5 mM luminol (5-amino-2, 3,-dihydro-1, 4-phthalazinedion) prepared in dimethyl sulfoxide was added to it. The sample was gently mixed by tapping and reading was taken in luminometer (Sirius, Berthold Detection Systems GmbH, Pforzheim, Germany) every 30 s for 15 min. PBS was used as internal control and same procedure was applied. The results were expressed as "Relative Luminescence Units per second per cell" (RLU/sec/cell).

**Cytochrome C oxidase activity**: 3 ml of citrated blood was used to assess the CCO activity. The peripheral blood mononuclear cells (PBMCs) were isolated using standard Ficoll-Hypaque density gradient centrifugation. The PBMCs were resuspended in 200 µl lysis buffer (10 mM Tris-HCl, pH 7.0, containing 250 mM sucrose and 1 mM n-dodecyl-β-D-maltoside) and active CCO was solubilized from mitochondria by adding N-dodecyl-β-D-maltoside. The lysate was stored at -80°C till further use. The CCO activity was measured by CCO Assay Kit (Sigma; Code: CYTOCOX1), strictly according to the manufacturer's instructions using Beckman Coulter spectrophotometer (DU-730). The final results were calculated as per the following formula.

$$\text{Units/ml} = \frac{\text{DA/min} \times \text{dil} \times 1.1}{(\text{Vol. of Enzyme}) \times 21.84}$$

A/min = A/minute (sample) - A/minute (blank)

dil = dilution factor of enzyme or sample

1. 1 = reaction volume in ml
21. 84 = $De^{mM}$ between ferrocytochrome c and ferricytochrome c at 550 nm

### Statistical analysis

Data were presented as number (%) or mean ± SD/median as approximate. Quantitative baseline variables were compared among the groups using one-way ANOVA and qualitative variables were compared using Chi-square test. The difference in median was compared using Kruskal–Wallis test followed by post-hoc Bonferronni correction.

## Results

No significant difference between the mean age in any of the three groups. ($p > 0.14$) was observed. The peripheral blood

indices (hemoglobin, hematocrit, RBC count, Leukocyte count, etc.) were comparable in all the groups. The demographic data of all the three groups along with the peripheral blood indices are given in Table 1.

A total of 233 participants were screened for this study (135 in Group I, 51 in Group II and 47 in Group III). In Group 1; out of 135 subjects, 13 had dementia, 3 had past history of stroke, 9 had head injury, 14 had uncontrolled dyskinesia, 9 were on antipsychotic medications. 7 did not consent to participate. In Group II; out of 51 subjects, 2 had past history of stroke, 3 had severe disability, 6 did not give consent. In Group III, out of 47 healthy controls, 2 had hypertension, 2 had diabetes and 3 did not agree to participate.

Therefore, a total of 160 participants were studied which included 80 PD patients (Group I), 40 PPS (Group II), and 40 HCs (Group III) [Figure 1]. In Group I, 71.2% were males and 28.7% were females. In Group II, 67.5% were males and 32.5% were females. Similarly in Group III, 55.0% were males and 45% were females. The mean age of Group I, Group II, and Group III patients was 57.8 ± 9.8, 63.2 ± 7.1, and 52.42 ± 7.2, respectively. Mean age of disease onset in Group I was 50 ± 12.9 years.

In PD patients, the mean age of disease onset was 50 ± 12.9 years. Right side was the most frequent affected side (60%) as compared to the left (35%). 5% of patients had bilateral involvement. A greater number of patients presented with tremor dominant symptoms (72.5%) as compared to akinesia (27.5%) with different frequencies of most disabling symptoms (Gait - 2.5%, akinesia - 3.75%, rigidity - 22.5%, and tremor 71.25%). Sleep pattern disturbance were observed in most of the patients (63.75%). The clinical features of PD cases are given in Table 2.

The ROS was found to be increased in both PD patients (Median: 14.1314.13 ± 29.5 RLU/s/cell as well as PPS patients (Median: 17.43 ± 15.91 RLU/sec/cell), indicating a role of ROS in both PD as well as PPS disease processes. However, this does not explicate the cause effect relationship between ROS and disease etiology. The ROS increase in PD patients was significantly higher than healthy controls ($P = 0.0029$). Also, ROS in PPS patients was significantly higher compared to healthy controls ($P = 0.0500$). PPS patients had higher levels of ROS as compared to PD patients but the difference was not statistically significant ($P = 0.84$). This pattern was, however, not reflected in the CCO activity. The ROS and CCO function findings are depicted in Figures 2, 3, and 4.

The CCO activity was found to be diminished in PD patients (Median: 0.025 ± 0.013 units/ml) and PPS patients (0.027 ± 0.008 units/ml). The decline in CCO activity was more prominent in PD patients compared to PPS patients. However, the difference in the CCO activity between PD and PPS patients was not statistically significant ($P = 0.620$). This indicates a reverse patters of ROS elevation and CCO dysfunction in PD and PPS. There was a significant difference in the CCO activity between PD patients and healthy controls ($P = 0.001$). Also, the CCO activity was significantly diminished in PPS patients as compared to healthy controls ($P = 0.001$). ROS and CCO levels in all the three groups are given in Table 3.

**Table 1: Demographic values and red cell indices**

| Variables | GpI (PD; *n*=80) | GpII (PPS; *n*=40) | GpIII (HC; *n*=40) | *P* |
|---|---|---|---|---|
| Age | 57.9±9.8 | 63.2±7.1 | 52.42±7.2 | 0.247 |
| Sex | | | | |
| M | 71.2% | 67.5% | 55.0% | 0.893 |
| F | 28.7% | 32.5% | 45% | |
| Hb | 12.4±2.1 | 13.0±1.8 | 13.26±1.90 | 0.681 |
| Hct | 38.5±5.9 | 41.1±5.2 | 39.5±7.5 | 0.10 |
| RBC | 4.6±1.1 | 4.6±0.6 | 4.8±0.5 | 0.34 |

## Discussion

PD is an idiopathic disorder though a variety of factors (aging, genetic predisposition, environmental toxins, bacterial, and viral infections, etc.) have been thought to play a role in etiopathogenesis.[16] In almost all forms of PD, oxidative stress has been considered to be one of the most important factors and common mechanism leading to dopaminergic neuronal apoptosis. The presence of higher levels of oxidized lipids, proteins, and nucleic acids in substantia nigra of PD patients lends support to this notion.[17] Oxidative stress-mediated PD etiopathogenesis is also supported by lower levels of reduced glutathione found in nigral neurons of PD patients.[18]

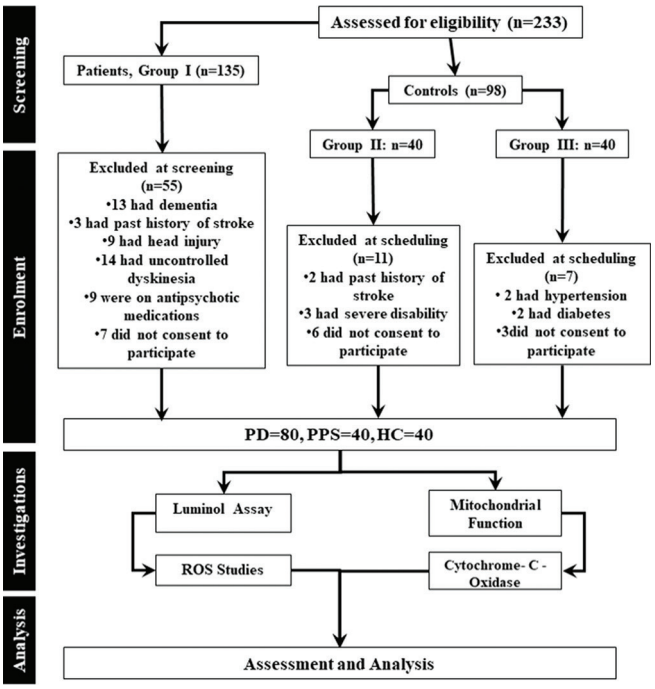


**Figure 1:** Study design in the form of a flow chart depicts four distinct stages of the study from patient recruitment through data acquisition and data analysis

**Table 2: Clinical features of PD Subjects**

| Variable | Results | | | |
|---|---|---|---|---|
| Age of Onset | 50±12.9 | | | |
| Side Affected | Right 60% (*n*=48) | Left 35% (*n*=28) | Both 5% (*n*=4) | |
| Affected | Upper Limb 78.75% (*n*=63) | Lower Limb 13.75% (*n*=11) | Both 6.25% (*n*=3) | Gait 1.25% (*n*=1) |
| Type of PD | Tremor 72.5% (*n*=58) | | Akinetic 27.5% (*n*=22) | |
| Most Disabling Symptom | Tremor 71.25% (*n*=57) | Rigidity 22.5% (*n*=18) | Akinesia 3.75% (*n*=3) | Gait 2.5% (*n*=2) |
| Sleeping Pattern | Normal 36.25% (*n*=29) | Intermittent 63.75 & (*n*=51) | | |
| Walking Pattern | Normal 40% (*n*=32) | Disturbed 60% (*n*=48) | | |
| Head Pain | Present 17.5% (*n*=14) | Absent 82.5% (*n*=66) | | |

**Table 3: ROS and CCO activity. *Results expressed in median±SD (RLU/sec/cell)**

| Variable | GpI (PD; *n*=80) | GpII (PPS; *n*=40) | GpIII (HC; *n*=40) | *P* (overall) | *P* | | |
|---|---|---|---|---|---|---|---|
| | | | | | I vs II | I vs III | II vs III |
| ROS | 14.13±29.5 | 17.43±15.91 | 7.53±15.58 | 0.0152 | 0.84 | 0.0029 | 0.0500 |
| Cyt C Oxidase | 0.025±0.013 | 0.027±0.008 | 0.117±0.049 | 0.0001 | 0.620 | 0.001 | 0.001 |

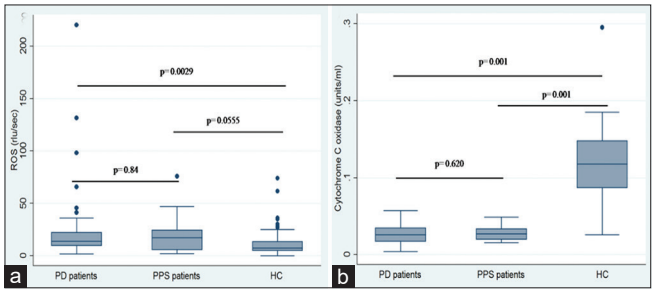


**Figure 2:** (a) Shows the values of ROS in PD patients, PPS patients in comparison to healthy controls. Elevation of ROS in both patient groups is elevated significantly. (b) Illustrates the mitochondrial dysfunction in terms of cytochrome C oxidase (CCO) activity. In both PD and PPS, CCO activity shows a statistically significant decline as compared to healthy controls. PD: Parkinson's disease, PPS: Parkinson plus syndrome, HC: Healthy controls, ROS: Reactive oxygen species

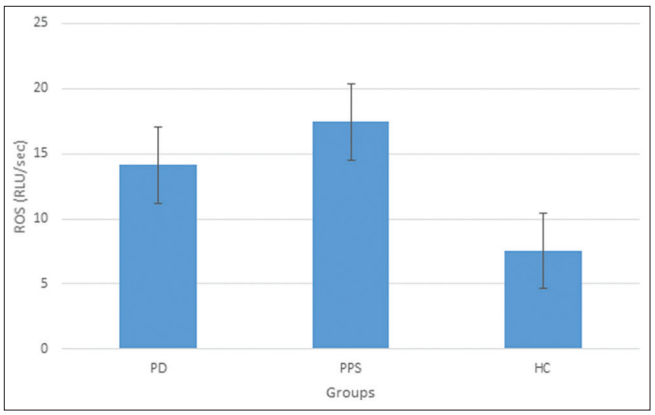


**Figure 3:** Shows the ROS levels in all the three groups

With this premise, our aim was to evaluate the oxidative stress, mitochondrial dysfunction, and peripheral blood indices in PD patients, PPS patients, and HCs in order to get an insight into the oxidative metabolism in etiopathogenesis of PD and related neurodegeneration. We actualized this objective by doing ROS estimation, evaluating mitochondrial health by CCO activity and checking peripheral blood indices. Peripheral blood indices [Table 1] were not deranged in PD and PPS in comparison to HCs except for mean corpuscular hemoglobin concentration (MCHC). ROS measurement and evaluation of CCO activity are two separate yet intricately linked markers. It is therefore desirable to deliberate upon as to why these two separate markers were checked in this study. To this effect, scientific literature has imbibed ample evidence indicating CCO dysfunction to be associated with increase in mitochondrial ROS production through multimodal regulation of CCO activity by a gamut of physiological and pathological (in case of ROS over production) factors.

According to some studies CSF and blood are true surrogate tissues for CNS metabolic status.[19] Also, there is ample evidence that peripheral blood shares considerable gene and protein expression patterns with CNS[20] justifying blood as a surrogate sample for CNS metabolism (in case of unavailability of the sample). The gene expression signature, mitochondrial status, and apoptotic gene expression pattern associated with PD pathogenesis are similar in peripheral blood and CNS.[20] Moreover, the genes that manage cellular bioenergetics and mitochondrial biogenesis are expressed in a closely similar pattern in blood cells and neurons.[21] We employed the Luminol method[22] because chemiluminescence is the most widely used and reliable technique for overall ROS estimation.[2,23]

ROS was found to be elevated in both PPS and PD patients, as compared to healthy controls. Elevation in ROS may indicate redox metal (more appropriately iron) toxicity (as can be interpreted in terms of MCHC elevation in our results), mitochondrial dysfunction (signposted by CCO), and microglial activation (microglial cells and blood monocytes belong to same

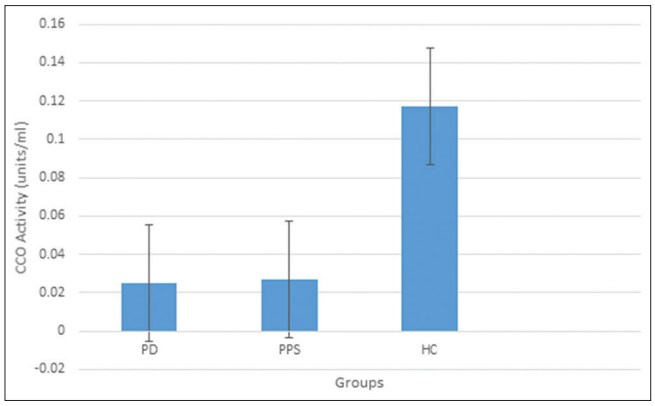


**Figure 4:** Shows the CCO activity in all the three groups

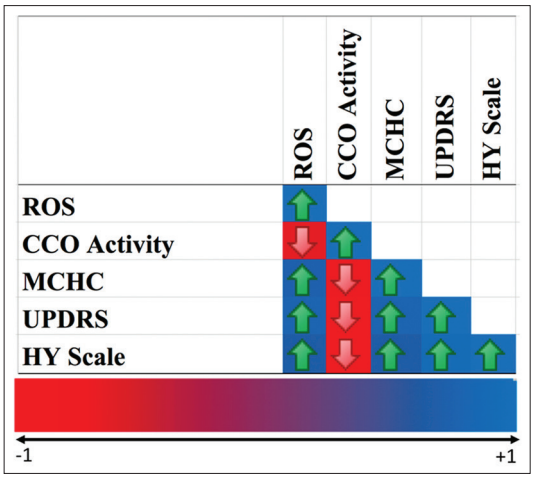


**Figure 5:** Shows the correlation between ROS levels, CCO activity, MCHC, UPDRS, H&Y Scale

category of cells). Elevated ROS activates certain important pathways of MAP-Kinase, excitotoxic calcium mobilization leading to neuronal apoptosis in PD.[24] Cellular bioenergetics involves utilization of glucose mediated through mitochondrial respiration by oxidative phosphorylation generating ATP.[25] We don't make any outright claim about gliosis in this study. One of the hypotheses that probably emerges is that mitochondrial dysfunction and CCO activity might also have some correlates with MCHC and, in turn, iron metabolism. Though the exact mechanism is not known and we have not been able to investigate this feature further, but brain MRI of these patients and iron deposition investigations would have added some further details. At this stage, it would be safe to conclude that peripheral blood cells show deranged MCHC in PD patients which may or may not reflect the picture in the CNS.

Neuron is energetically most expensive cell and there is consistently higher need of ATP synthesis which leads to the collateral production of $H_2O_2$ and other free radicals. Any mitochondrial insult can lead to dramatically higher production of ROS. This elevation of oxidative stress leads to peroxidation of cardiolipin causing of Cytochrome-C leakage to the cytosol, thereby, eliciting apoptosis.[26] Since the dopaminergic neurons have higher ability to generate ROS, a slight derangement can lead to harmful effects and mitochondrial ETC damage with leakage of electrons causing further ROS elevation. This indicates that mitochondrial dysfunction has special correlation to PD as compared to other neurodegenerative diseases.

Loss of Complex–I activity has already been shown to be associated with PD.[27] But, to the best of our knowledge, the present study is the first to report reduction in CCO activity (in blood), which is a part of complex-IV of the mitochondrial ETC. This specifies that derangement in ETC anywhere throughout the process is toxic to dopaminergic neurons. Recently reported higher proportion of defective mitochondrial respiratory chain in dopaminergic neurons of PD patients[28] lends support to our results. Mutations in genes already implicated in PD have been shown to be associated with mitochondrial dysfunction.[29] Our findings are in overall agreement with the already published literature associating mitochondrial dysfunction with PD pathogenesis through ROS elevation.[30,31] However, this study adds an important opinion that complex-I is not the only insult point in mitochondrial respiratory chain that predisposes to PD but complex-IV may very well be implicated. This finding is likely to help in understanding PD pathogenesis better. It is interesting to observe that our results indicate imperative alterations in key function of the cells in peripheral blood. CCO catalyses the final step in ETC and also interacts with complex I and III to form the, what is referred to as, respirasome and hence exerts control on mitochondrial function. It can, therefore, be speculated that primary loss of CCO may precipitate secondary effects on respirasome assembly. Such a phenomenon may elicit certain intricate biochemical phenotypes which in certain cases may be therapeutic target in terms of the use of antioxidants in PD patients and patients suffering from psychiatric disease with etiological basis in mitochondrial dysfunction.

Peripheral blood indices [Table 1] were not deranged in PD and PPS in comparison to HCs except for mean corpuscular hemoglobin concentration (MCHC). This additional interesting finding has not been reported by any other study in the literature. The finding prompts us to further this study toward details of iron metabolism and its correlation with oxidative stress and tamitochondrial dysfunction [Figure 5]. Imaging studies may also be initiated to evaluate brain iron metabolism in PD. This finding can have many interpretations but the one that is pertinent is that mitochondria may play pivotal role in iron metabolism of vice versa. If higher MCHC indicates iron overload, then (i) iron metabolism, (ii) oxidative stress, and (iii) mitochondrial dysfunction forms a triad of pathogenetic mechanisms for PD, which warrants further research. Mitochondria are involved in the synthesis of heme and iron-sulphur clusters that are vital to proper neuronal function.[32] Mitochondria house the iron pool which is active from redox point of view.[33] A slight increase in the redox pool leads to mitochondrial dysfunction and changes in membrane permeability.[33] Any excess in the iron pool leads to higher production of hydroxyl radicals (vital component of ROS). This leads to elevation in ROS and consequently

mitochondrial dysfunction. In the course of this progression, the impact of the vicious triangle of ROS, mitochondrial dysfunction, and iron overload increases precipitating PD [Figure 1]. Imaging studies, to this effect, are justified to verify any iron deposition in PD brains in our cohort. Biopsies taken from PD cadaveric brains (particularly substantia nigra) may be helpful in getting a useful and meaningful insight. This objective would be the rationale of our future studies.

This study is the first probe toward evaluation of CCO activity in blood of PD patients which provides a cogent imperative role of mitochondrial etiology and oxidative stress. The unique incidental observation of deranged MCHC values in PD patients is an important and thought-provoking strength of this study and bolsters the importance of iron metabolism in PD pathology. This, as a consequence, forms the new research questions for our next step in this direction.

There is no cure for PD except for symptomatic management in early cases. A thorough understanding of the molecular and cellular mechanisms involved in PD etiopathogenesis is, therefore, indispensable. Since oxidative stress is central to major pathogenic mechanisms of PD mediated through mitochondrial dysfunction and iron metabolism (as depicted in Figure 2), we thought of probing all these areas in a single study to get a coherent and multidimensional insight into the pathogenic mechanisms that will help in designing relevant clinical therapies for this disorder. The present study might be considered as the preliminary probe into the triad of above discussed molecular syndromes suggesting an interplay between mitochondria, iron metabolism, and ROS in dopaminergic neuronal apoptosis. These findings also brings rationale in antioxidant therapies targeted to the entire mitochondrial complex to address PD. Manipulation of iron metabolism is also a potential target for design of therapies for PD. It looks like a safe hypothesis to conclude that that ROS elevation in PD and PPS may have different mechanisms thereby confirming them to have different etiopathologies and providing an explanation of the observation of different response to therapies. However, a caveat to mention here would be that mitochondrial ETC complex deficit (including those of complex IV) have been reported to be associated with pathophysiology of certain psychiatric disorders in addition to various neurodegenerative conditions like AD and PD. So mitochondrial dysfunction may not enjoy a specificity with PD only. It is important to recognize that molecular basis of different clinical presentations due to CCO deficiency remains unexplained. In that context, it would be ideal to classify the PPS patients according to MSA, PSP, CGBD, and DLBD and also subgroup the PD patients within the purview of clinical manifestations, individual specific variable course and disease severity.

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## Key Messages

1. Mitochondrial dysfunction and oxidative stress is associated with PD and PPS.
2. Mitochondrial dysfunction and oxidative stress may play an important role in etiopathogenesis of PD.
3. Addressing oxidative stress and mitochondrial damage may serve as an adjunctive therapy for PD and PPS.
4. Iron metabolism as reflected in the red cell indices may aid in differentiating PD from PPS.

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## Conflicts of interest

There are no conflicts of interest.

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