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Plasma level of nitrates in patients with Parkinson's disease in West Bengal

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Abstract

Background: Oxidative stress is implicated as a major factor for nigral neuronal cell death. It has been suggested that nitric oxide (NO) might be responsible in the neuronal degeneration of substantia nigra compacta in patients with Parkinson's disease (PD). *Methods:* To elucidate the possible role of NO as a risk factor for PD, we determined the plasma levels of nitrate in 80 PD patients and 80 age and sex-matched controls. *Results:* There was a significant difference between the mean plasma nitrate level of PD patients (34.5 ± 4.2 μmol/l) vs. controls (31.8 ± 3.15 μmol/l) (P<0.001). Moreover, there was a positive correlation in PD patients between plasma nitrate level and age, age at disease onset, duration of disease, Hoehn and Yahr stage, and Unified Parkinson's Disease Rating Scale (UPDRS) score. However, PD patients with short disease duration did not have elevated plasma nitrate levels compared to control subjects.

*Conclusion***:** Our results argue against the hypothesis that NO is important in the development of PD in West Bengal.

INTRODUCTION

Parkinson's disease (PD) is a common, idiopathic, neurodegenerative disorder that produces bradykinesia, muscular rigidity, rest tremor and loss of postural balance. The cardinal pathologic change of Parkinson's disease is the degeneration of dopaminergic neurons in the substantia nigra pars compacta. The exact cause of nigral neuronal death in Parkinson's disease is still unknown; however, oxidative stress[1], mitochondrial respiratory failure[2-4] and increase in iron concentration[5,6] have been implicated as major contributors. Free radicals are thought to be produced locally within the basal ganglia and lead to progressive degeneration and ultimately death of dopaminergic neurons in susceptible individuals. The most common cellular free radicals are hydroxyl radical ($OH^{\cdot}$), superoxide radical ($O2^{\cdot-}$) and nitric oxide ($NO^{\cdot}$). Other molecules such as hydrogen peroxide (H_2O_2) and peroxynitrate ($ONOO^-$) are not free radicals, but can lead to the generation of free radicals through various chemical reactions.

NO plays an essential role in brain vasoregulation and neurotransmission.[7,8] In excess, NO can inhibit cytochrome oxidase in mitochondria, leading to increased linkage of electrons and more O_2^- formation.[9] NO can react with O_2^- to give peroxinitrate[10], ($ONOO^-$): $NO^- + O_2^- \rightarrow ONOO^-$. As a signal molecule, NO plays an important role in a variety of signal transduction pathways that are crucial for maintaining the physiologic functions of vascular, respiratory, immune, muscular, and nervous systems. NO and its derivatives are also involved in the pathogenesis of various types of diseases including, but not limited to, neurodegenerative disorders. Although the molecular mechanisms of how NO contributes to disease are not completely understood, studies have shown that NO may cause neuronal injury and death by mediation of excitotoxicity, damage of DNA, and/or modification of proteins.[11]

The implications of NO in neurotoxicity prompted us to focus on its role in degenerative neurological diseases. In the present study, we determined the plasma levels of nitrates (an indirect indicator of NO synthesis) in PD patients and a control cohort. The objective of this study was to determine whether alterations in plasma nitrate level could be associated with the risk of developing PD in West Bengal, India. To the best of our knowledge, no such studies have been carried out in this region.

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METHODS

The study protocol was approved by the Ethics Committee of the Institute and collaborating hospitals. The patient cohort consisted of 80 patients with idiopathic PD without a family history (61 males, 19 females) selected from the Bengali-speaking population in West Bengal visiting the outpatients neurology department of Calcutta Medical College and Hospital and the National Neuroscience Centre (NNC), Kolkata. Clinical data and detailed family history of each patient was collected with the help of collaborating clinicians. The Unified Parkinson's Disease Rating Scale (UPDRS)[12] and Hoehn and Yahr staging[13] were performed to quantify disease severity.

The control group consisted of 80 healthy community-based, age and sex-matched volunteers (68 males, 12 females), residing in the same areas and from the same ethnic background as the PD patients (P>0.05). None of the controls had any diagnosable neurological disorders.

Operational definitions

All PD patients recruited met the following criteria (at the time of diagnosis and within the study period): 1) The presence of at least three of the following signs: resting tremor, cogwheel rigidity, bradykinesia and postural reflex impairment, at least one of which must be either rest tremor or bradykinesia[14]; 2) No suggestion of secondary parkinsonism due to drugs, trauma, brain tumor or treatment within the last 12 months with dopamine-blocking or dopamine-depleting agents; and 3) No atypical features such as prominent oculomotor palsy, cerebellar signs, vocal cord paresis, severe orthostatic hypotension, pyramidal signs, amyotrophy or limb apraxia.

The following exclusion criteria were applied both to the PD and control groups[15-18]: 1) Therapy with nitrovasodilators or glucocorticoids in the last six months; 2) Ethanol intake higher than 80 g/day during in the last six months; 3) Previous history of haematologic or autoimmune disease, chronic hepatopathy, renal failure or severe systemic disease; and 4) Atypical dietary habits, including diets rich in nitrates or calcium.

Collection of blood samples and plasma separation

Approximately 3 mL of peripheral blood was collected in a Vacutainer containing K2 EDTA (Becton Dickinson Vacutainer system), with written informed consent from all subjects. Blood was centrifuged at 3000 rpm for 8 minutes. Plasma was separated from the buffy coat carefully and stored at 4°C until analysis. NO is difficult to analyse because of its very short half-life. The measurement of the oxidation products, nitrite and nitrate, provides a useful and indirect estimation of NO concentrations.[19-,21] We measured plasma nitrate using a kinetic method where nitrate is reduced to nitrite (Greiss' reaction) by copper-coated cadmium granules[22] with minor modifications and quantification carried out in a Perkin Elmer Spectrophotometer (Lambda 25 UV/VIS Spectrometer)(wavelength λ=540 nm). All analysis was carried out within 72 hours of blood collection.

Statistical analysis

The results are expressed as mean ± standard deviation (SD). Statistical analysis included the two-tailed Student's t-test, one-way analysis of variance (ANOVA) and Pearson's correlation coefficient (r), using SPSS v11.5 software. A P-value of less than 0.05 was considered statistically significant.

RESULTS

Clinical and demographic data of PD and control subjects are summarized in Table 1. The age (P=0.74) and sex (P=0.23) distribution of patients and controls was similar. More than three-fourths of each group comprised males. All except four PD patients were treated with antiparkinsonian medication. The mean plasma level of nitrates differed significantly between PD patients and the control cohort (34.5 ± 4.2 μmol/l and 31.8 ± 3.15 μmol/l, respectively, t=4.73; df =79; P<0.001). However, there was no significant difference in plasma nitrate level between the subgroup of PD patients with disease duration of two years or less vs. that of the control group (P=0.0531). In the PD group as a whole, there was a positive correlation of nitrate levels with patient age (r=0.35; P=0.0014), age at disease onset (r=0.26, P=0.0191), disease duration (r=0.731, P<0.0001), UPDRS (r=0.407, P=0.0002), and the Hoehn and Yahr stage (r=0.412, P=0.00015). An ANOVA showed that plasma nitrate levels differed significantly with the increase in disease duration (F=65.61, P<0.0001). Analysed in a different way (see Table 1 for P values), NO levels were higher in PD patients with disease onset > 50 years, and in those with a greater degree of parkinsonism (UPDRS score > 30 or Hoehn and Yahr > 2). There was no significant

Table 1: Demographic and clinical characteristics of PD and control subjects

	Patients (n = 80)	Controls (n = 80)	P value
Age (yrs)	58.2 ± 12.2	57.6 ± 9.1	*P*=0.74
Male : Female	61:19	68:12	*P*=0.23
Age of onset of PD (years)	55.3 ± 5.2		
Duration of PD (years)	3.6 ± 1.6		
UPDRS scores			
Total for parts I-III (items 1-31)	31.2 ± 5.2		
ADLs scale (items 5-17)	13.8 ± 1.8		
Motor scale (items 18-31)	14.9 ± 2.1		
Hoehn and Yahr stage	2.4 ± 1.1		
Plasma nitrate levels (μmol/l)			
Total nitrate level (μmol/l)	34.5 ± 4.2	31.8 ± 3.15*	*P*<0.001
Age of onset (years)			
≤50	29.7 ± 5.1		*P*<0.0001
51-70	39.6 ± 6.3		
Duration of the disease (years)			
≤ 2	30.2 ± 1.35		
2-5	33.3 ± 1.28		
≥ 5	38.3 ± 3.68		
UPDRS			
≤ 30	31.5 ± 3.67		*P*<0.0001
> 30	36.7 ± 2.50		
Hoehn and Yahr stages			
≤ 2	30.9 ± 2.71		*P*<0.0001
> 2	37.8 ± 3.83		

correlation between plasma nitrate level and age in the control group (r=-0.06).

DISCUSSION

Mechanisms underlying neuronal death in PD are poorly understood, although several *in vitro* studies have suggested the involvement of oxidative stress.[23] The concept that oxidative stress occurs in PD derives primarily from the realization that the metabolism of dopamine, by chemical or enzymatic means, can generate free radicals and other reactive oxygen species through autoxidation and dopamine oxidation by monoamine oxidase B. A free radical gas, NO plays multiple roles in the brain as a neuronal messenger molecule, a regulatory factor of cerebral circulation, and in host defense. NO is synthesized from the amino acid L-arginine by NO-synthase (NOS). On the other hand, NO is neurotoxic if produced in excess.[24] In degenerative neurological diseases, NOS-containing neurons preferentially survive. The remaining NOS neurons can be the source of NO that mediates neurotoxicity.

According to the hypothesis of Youdim *et al*. 1993[25], NO could be implicated in the pathogenesis of PD in at least three ways: NO-mediated involvement of corticostriatal glutamatergic neurons[26,27]; NO interaction with the iron storage protein ferritin resulting in the release of iron, formation of iron-nitrosyl complexes and promotion of free radicals, triggering lipid peroxidation[28]; and NO-induced impairment of mitochondrial function.[29,30] Thus, NO could damage the iron-dependant mitochondrial NADH-

coenzyme Q reductase in a similar way to that described in parkinsonian substantia nigra.[31]

Studies on nitrite and nitrate measurements in the cerebrospinal fluid (CSF) of PD patients have been contradictory, showing no difference[32,33], higher[34,35] or lower[36,37] levels compared to controls. Tuncel *et al*.[38] found that the serum NO level of PD patients was significantly reduced compared to that in controls; in this study, PD patients had a mean disease duration of 4.9 ± 3.1 years. Some studies showed that CSF and plasma nitrate levels did not correlate with age at PD onset, disease duration, or scores of parkinsonism severity.[39,40]

Results of the present work indicate that plasma nitrate levels are higher in PD patients than in controls. However, this difference does not justify stating that plasma nitrate levels are therefore related to the risk of developing PD, particularly as patients with short PD duration did not have elevated levels of NO. In conclusion, the present data suggest that plasma nitrate concentrations are not related to the risk of developing PD in West Bengal. However, the question of cause and effect can best be answered by a longitudinal study, which our study lacks.

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