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Nitric oxide and peroxynitrite serum levels in Parkinson's disease: correlation of oxidative stress and the severity of the disease

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Abstract. – BACKGROUND: Nitric oxide (NO) and its toxic product peroxynitrite contribute to oxidative stress and neurodegeneration in Parkinson's disease (PD). The relationship of serum levels of these oxidants with the severity of the disease [evaluated by the Unified Parkinson's Disease Rating Scale (UPDRS)] is not established.

AIM: This study was designed to evaluate whether patients with PD had higher NO and peroxynitrite serum level or not.

PATIENTS AND METHODS: Fifty eight patients with PD and 15 healthy volunteers entered this study. The concentrations of serum NO and peroxynitrite were assayed and their correlation with the UPDRS score was assessed.

RESULTS: Mean serum NO levels in patient group was 29.8 ± 21.631 versus 7.49 ± 2.573 in control group, which was significantly higher in patients ($p \leq 0.0001$). Peroxynitrite levels in patient and control groups were 7.37±3.501 µmol/L and 3.94 ±1.389 µmol/L respectively. Patients had a significantly higher peroxynitrite level ($p = 0.0004$).

CONCLUSIONS: Higher levels of NO and peroxynitrite leads to higher UPDRS scores. It seems since current PD treatments do not affect the pathology of the disease, using drugs that exert neuroprotective properties should be considered for the treatment of PD in order to prevent further neuronal cell loss.

Key Words:
Parkinson's disease, Nitric oxide, Peroxynitrite, UPDRS, Neuroprotection.

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder that progressively affects motor system as well as neuropsychiatric features including tremor, rigidity, bradykinesia, cognitive dysfunction, dementia, mood disorders. Frequency of PD is about 0.3% in the general population and more than 1% in those over 60 years old. Race and geographic region do not influence PD[1,2]. A major hypothesis for the pathogenesis of the disease is the neurotoxicity caused by free radicals leading to neuronal cell loss through overproduction of reactive oxygen and nitrogen species[3].

Nitric oxide (NO) is generated by nitric oxide synthetase and high concentrations or prolonged exposure to NO promotes a deleterious inflammatory response leading to secondary tissue necrosis and cell apoptosis. Damage to dopaminergic neurons in brain is one of the underlying mechanisms in PD that finally results in disability of patients.

Rapid reaction of NO with superoxide anions yields the potent oxidant peroxynitrite ($ONOO^-$) which leads to formation of hydroxyl radicals and nitrogen dioxide resulting in unwanted side effects of elevated NO[4]. Overproduction of these oxidants overwhelms antioxidant defense of the body. Hence, antioxidant use to restore this balance is a major therapeutic intervention for cell damage prevention[3,5,6].

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Moreover. a 50% neuronal loss at the onset of PD is another factor that supports the idea of preventing further cell death by neuroprotection[7].

Pharmacotherapy for PD improves patients' symptoms, but its influence on neuroprotection has not yet been established and currently none of these medications are known to be neuroprotective[8,9]. Based on different studies NO and peroxynitrite have a role in the etiology of PD, but their effect on the severity and progression of this disease has not been confirmed[4,10-12].

The Unified Parkinson's Disease Rating Scale (UPDRS) was used as a score to determine the severity of the disease (where the higher the score is the more severe the disease is). It has four components (mentation, behavior and mood, activities of daily living, motor, complications) and is an efficient tool for evaluating all aspects of Parkinson's disease and its related disabilities. It is widely used as a gold standard scale in different clinical trials for monitoring the progression and treatment response in PD patients[3,13-16].

Our aim is to assess the correlation between serum levels of NO and peroxynitrite with the UPDRS score of patients suffering from PD.

Patients and Methods

We conducted a case-control study at two sites one being the Neurology Outpatient Clinic at Roozbeh Hospital [affiliated to Tehran University of Medical Sciences (TUMS)] and the other being a private Center mainly focused on geriatrics neurology in Tehran, Iran from April 2010 to March 2011.

The study was permitted by the TUMS Ethics Committee. Patients were all informed and proper consent forms were filled by each patient same as each volunteer of the control group.

Inclusion criteria were having Parkinson's disease, being able to participate in the UPDRS assessment and fulfilling the consent form.

The control group constituted of healthy volunteers with matched demographic characteristics. They didn't have any family history of Parkinson's disease and the physical examinations were all normal.

The exclusion criteria for both patients and control volunteers were major diseases or states and major drugs, supplements or specific diet that affected NO production including concomitant disease such as rheumatoid arthritis (RA), diabetes mellitus, chronic kidney disease, any malignancy and history of recent myocardial infarction and stroke. Patients with definite diagnosis of Alzheimer's disease (AD) were excluded since there are studies showing the over production of nitric oxide and peroxynitrite in AD[3]. None of the subjects received following drugs or supplements: nitrates, statins and steroids, phosphodiesterase inhibitors such as sildenafil, pentoxifyllin, N-acetyl cystein, insulin, Vitamin E, Vitamin C, Ginko biloba, melatonin or Coenzyme Q 10. Cigarette smoking and strict vegetarian diet were other exclusion criteria. Since different pathogenesis pathways might be affecting atypical forms of PD (for example Progressive Supranuclear Palsy) we excluded these patients as well[3,17-19].

Laboratory tests including blood glucose, lipid profile, uric acid, thyroid function test, erythrocyte sedimentation rate (ESR), creatinine and complete blood count (CBC) were assessed for all subjects at the beginning.

To rate the severity of the disease, each patient was assessed by the UPDRS[10].

First this scale was translated to Farsi (Iran's official language) by a neurologist expert in geriatrics and a pilot survey was done to assess possible incompatibilities or need for special adjustments. A clinical pharmacy resident (one of the authors) was trained by a neurologist expert in geriatrics to perform some of the UPDRS evaluations.

Every patient or volunteer was assessed individually and the process of the UPDRS assessment took about 30 minutes for each person.

A patient data form (attached to the paper) was also designed to accurately gather all the information necessary for the study. It concluded name, telephone, address, demographic characteristics, medical history, drug history, family history, current medications, smoking and type of diet.

At the same day of the UPDRS evaluation venous blood samples were taken from each subject and the sera were separated by centrifugation (3000 rpm for 3 min) and were stored at -80°C until the time of NO and peroxynitrite assay (the maximum time a sample was kept at this condition was 4 months).

NO goes under several chemical reactions in the serum and its final products are nitrate and nitrite. Griess method is a known way for nitrate/nitrite assay and when concentrations are high enough to be shown by mol/L the result of this assay is comparable to other methods of nitrate/nitrite measurement such as HPLC[20,21]. The samples' NO content were measured by

nitrate/nitrite colorimetric assay kit (Cayman®, Ann Arbor, MI, USA). The colorimetric absorbance was read at 540 nm by an ELISA plate reader (Synergy, BioTek®, Winooski, VT, USA). Serum NO was then calculated in respect to standard nitrate absorbance concentration curve according to assay protocol.

Serum peroxynitrite was assessed using a novel method for detecting reactive oxygen species (ROS) using hydroxyphenyl fluorescein (HPF) probe developed by Setsukinai et al[22]. This method is a novel one step assay for peroxynitrite detection. When HPF reacts with ROS such as peroxynitrite or hydroxyl radical it shows a strong dose dependent fluorescence. Peroxynitrite level was measured using hydroxyl radical (OH), and peroxynitrite ($ONOO^-$) detection Kit (Cell Technology, Mountain View, CA, USA). The fluorescence intensity was then measured by an ELISA plate reader (Synergy, BioTek®, Winooski, VT, USA) at a utilizing excitation of 488 nm and an emission of 515 nm. The concentration of peroxynitrite was then calculated in respect to a standard solution of peroxynitrite and the fluorescence increase-concentration curve[23].

This standard solution was synthesized according to the following description: same amount of 0.6 $molL^{-1}$ of sodium nitrite and 0.7 $molL^{-1}$ H_2O_2 containing 0.6 $molL^{-1}$ HCl were mixed rapidly and then quenched with equal volume of 1.5 $molL^{-1}$ NaOH solution. The excess H_2O_2 was removed with MnO_2 powder. The final concentration of peroxynitrite was determined by a UV spectrophotometer (SPEKOL® 1300/1500, Analytik Jena AG, Jena, Germany) at 302 nm.

Statistical Analysis

All data were expressed as mean ± standard deviation (SD) of mean. The normality of data on NO and peroxynitrite levels and UPDRS among patients and control group was first determined by Kolmogorov-Smirnov test (KS-test).

The correlation of serum NO and peroxynitrite levels with the UPDRS score were compared by Pearson correlation test. Significance was defined as a p value of < 0.05.

Results

A total of 77 subjects were enrolled in this study of which 58 of them were in patient group and 15 in control group. Four patients were dropped out of the study since they had medical conditions which were among exclusion criteria and were not reported in the data form. All patients' Parkinson's disease was confirmed by a neurologist expert in geriatrics.

Table I shows data about age, gender, duration of disease and type of drug therapy. All patients were in normal body mass index (BMI) range (18.5 to 24.9 kg/m^2). There was no significant difference in demographic characteristics between patients and control group.

Only 37.9% of the patients were on a single drug regimen. 41.4% were on two drugs, 19.0% and 1.7% were on three and four drugs respectively.

As presented in Tables II and III, we found that there is no significant difference between serum levels of NO and UPDRS with sex nor age (p values for correlation of NO levels with sex and age are 0.804 and 0.166 respectively. These value for UPDRS correlations are 0.886 and 0.225) but peroxynitrite levels show to be higher in men and also have a positive correlation with age (p values for correlation with sex and age are 0.026 and 0.007 respectively).

Table I. Demographic data of patients and control group.

	Patient group (number = 58)	Control group (number = 15)
Sex	F: 43.9% M: 56.1%	F: 60% M: 40%
Age (years)	64.4 ± 11.1 (mean ± SD)	58.3 ± 10.0 (mean ± SD)
Duration of disease (years)	5.4 ± 3.7 (mean ± SD)	–
Type of drug therapy for PD	Patients on Levodopa: 75.9% Patients on dopamine agonists: 31.0% Patients on amantadine: 50.0% Patients on seligiline: 15.5% Patients on propranolol: 12.1%	–

Table II. Relationship between sex with serum levels of NO and peroxynitrite and UPDRS in patients and control group.

		Number	Mean ± SD	*p*-value
UPDRS	Female	25	28.44 ± 16.76	0.886
	Male	32	29.13 ± 18.97	
Nitrate level	Female	25	29.41 ± 18.82	0.804
	Male	32	30.86 ± 23.78	
Peroxynitrite level	Female	25	6.33 ± 2.16	0.026
	Male	32	8.27 ± 4.13	

p: Level of significance (< 0.05), SD: Standard Deviation.

In terms of duration of disease, longer duration correlated significantly with higher UPDRS and NO levels (*p* values are 0.007 for UPDRS and 0.017 for NO levels) but this correlation wasn't significant about peroxynitrite levels (*p* value: 0.102).

The mean NO level was 29.8 ± 21.631 mol/L in patients and 7.49 ± 2.573 mol/L in control group, while peroxynitrite level was 7.37 ± 3.501 mol/L and 3.94 ± 1.389 mol/L respectively.

The mean UPDRS score was 28.48 ± 17.916 in patients versus 0.47 ± 1.06 in control group.

Table IV shows serum levels of NO and peroxynitrite and the UPDRS score in the two groups.

Patients with Parkinson's disease had significantly higher serum levels of NO and peroxynitrite in comparison to control group (*p* values are ≤ 0.0001 for NO and 0.0004 for peroxynitrite). The UPDRS score of patients was significantly higher than control group (*p* value: ≤ 0.0001). Table V shows the correlation between serum levels of these factors and the UPDRS score in patients.

Discussion

The current study has shown that higher serum levels of NO and peroxynitrite in patients showed positive correlations with the UPDRS scores. The role of oxidative and nitrosative stress in pathogenesis and etiology of PD has been established and this positive correlation supports the hypothesis of their role in the progression of PD.

Oxidative stress leads to damage to dopaminergic neurons due to impaired mitochondrial function, alterations in brain iron content and change of antioxidant protective systems (mostly superoxide dismutase and reduced glutathione). The exact nature of cell death due to specific oxidants in PD remains unknown, but there are evidences of hydroxyl radical, peroxynitrite and nitric oxide role. The formation of peroxynitrite is depended on NO content[11,16].

The Unified Parkinson's Disease Rating Scale was used in this study for evaluation of the severity of PD. This scale can be used as the standard tool for evaluating patients with PD in Iran since it is flexible and not affected by education, culture, race nor geographical region. It also covers all PD related impairments including motor disabilities and mental problems[13,14].

Some studies reported the correlation of cerebrospinal fluid (CSF) levels of NO and with the severity of disease. In one study[24] the results were consistent with our findings; higher UPDRS scores were related to higher CSF

Table III. Relationship between age and duration of disease with serum levels of NO and peroxynitrite and UPDRS in patients and control group.

	Pearson correlation	*p*-value
Correlation between age and UPDRS	0.10	0.225
Correlation between age and NO levels	0.13	0.166
Correlation between age and peroxynitrite levels	0.32	0.007
Correlation between duration of disease and UPDRS	0.32	0.007
Correlation between duration of disease and NO levels	0.28	0.017
Correlation between duration of disease and peroxynitrite levels	0.17	0.102

p: Level of significance (< 0.05).

Table IV. Serum levels of NO and peroxynitrite and the UPDRS score in normal controls and patients with PD.

		Number	Mean ± SD	*p*-value
UPDRS	Control	15	0.47 ± 1.06	≤ 0.0001
	Patients	58	28.48 ± 17.92	
Nitrate level	Control	15	7.49 ± 2.57	≤ 0.0001
	Patients	58	29.80 ± 21.63	
Proxynitrite level	Control	15	3.94 ± 1.39	0.0004
	Patients	58	7.37 ± 3.50	

p: Level of significance (< 0.05), SD: Standard Deviation.

NO levels. Shukla R et al[25] found that NO content of CSF in patients with PD had been higher compared to control group; however, this finding was not significant. This might be due to the fact that in that study the control group suffered from central or peripheral neurological problems but in the present study the control group was selected among healthy volunteers. The study also reported no correlation between NO CSF levels with duration of disease, type of drug therapy for PD, age or gender. The present investigation found NO levels do not have any correlation with sex or age. However, longer duration of disease was correlated with higher NO levels[25]. In addition taking CSF samples is an invasive procedure which causes great discomfort for patients. Hence, this work was designed to examine the levels of serum oxidative biomarkers due to the fact that taking a blood sample is convenient, patient friendly and less expensive.

Tuncel D et al[11] measured NO serum concentrations of 25 patients with PD using Griess method and the mean level was 2.3 ± 0.4 mol/L which was significantly lower than control group. The Authors of that research noted that this finding might be associated with adaption of NO and protective mechanisms in PD. This result is in contrast with our findings.

There are no prior investigations on measuring serum peroxynitrite levels in patients with PD and its relationship with the UPDRS scores. Peroxynitrite is a byproduct of NO and its level is important in disease progression.

The mean UPDRS of patients was 28.48 ± 17.92, this is mostly because of the fact that patients were selected from outpatient setting and had mild to moderate PD and severe cases were not included[10,16].

The medication mostly used among our patients was levodopa but this drug is not an ideal agent since it does not affect the mechanism disease progression[9].

Studies like the present report help us have a better understanding of all aspects of pathogenesis of PD and hence taking in account drug therapies that exert neuroprotective effects. These studies may suggest a new line of medications to be added to the conventional treatments for PD.

Conclusions

Our data indicate that elevated serum levels of NO and peroxynitrite have an impact on progression of PD. Levels of NO and peroxynitrite may be considered as biomarkers for PD progression in the future. However, other studies are necessary for definite recommendations.

It should be noted that at the present time no specific can be recommended for neuroprotection and controlling the progression of PD.

Table V. Correlation between serum levels of NO and peroxynitrite and the UPDRS score in patients.

Correlation between UPDRS and:	Number	Pearson correlation	*p*-value
NO level	58	0.89	≤ 0.0001*
Peroxynitrite level	58	0.51	≤ 0.0001*

p: Level of significance (< 0.05).

Patient Data Form. All fields must be completed.

1. Contact information
Date:
Name:
Telephone:
Address:

2. Demographic data
Gender: M/F
Date of Birth:
Level of education:
Case or Control:

3. Patient History
Duration of Parkinson's disease (for patients): (yrs)
UPDRS score:
Other medical conditions: Hypertension, Diabetes mellitus, Rheumatoid arthritis, Chronic kidney disease, Alzheimer's disease, Stroke malignancy, Myocardial infarction, others
Family history of Parkinson's disease: Laboratory data: Blood sugar, Creatinine, Lipid profile, Thyroid function test, CBC Uric acid, ESR

4. Drug therapy
Drugs for Parkinson's disease (name of the drug/daily dose):
Levodopa:
Dopamine agonists:
Selegiline:
Amantadine:
Others:

5. Drug history/habits/special diets
Other drugs or supplements:
Smoking:
Vegetarian diet:

Name/Signature of the study partner in charge of filling the form.

References

1) Jankovik J. Etiology and pathogenesis of Parkinson disease. In: Basow D, editor. Waltham, MA: Up to date; 2010.
2) Chou K. Clinical manifestations of Parkinson disease. In: Basow D, editor. Waltham, MA: UpToDate; 2010.
3) Maccioni R, Muoz J, Barbeito L. The molecular bases of Alzheimer's disease and other neurodegenerative disorders. Arch Med Res 2001; 32: 367-381.
4) Jomova K, Vondrakova D, Lawson M, Valko M. Metals, oxidative stress and neurodegenerative disorders. Mol Cell Biochem 2010; 345: 91-104.
5) Ascenzi P, di Masi A, Sciorati C, Clementi E. Peroxynitrite–An ugly biofactor? Biofactors 2010; 36: 264-273.
6) Stern MB. The early treatment of Parkinson's disease: levodopa, dopamine agonists or both. Parkinsonism Relat Disord 2001; 7: 27-33.
7) Bjorklund A, Kirik D, Rosenblad D, Georgievska B, Lundberg C, Mandel R. Towards a neuroprotective gene therapy for Parkinson's disease: use of adenovirus, AAV and lentivirus vectors for gene transfer of GDNF to the nigrostriatal system in the rat Parkinson model. Brain Res 2000; 886: 82-98.
8) Park S, Geddes TJ, Javitch JA, Kuhn DM. Dopamine prevents nitration of tyrosine hydroxylase by peroxynitrite and nitrogen dioxide. J Biol Chem 2003; 278: 28736-28742.
9) Suchowersky O, Gronseth G, Perlmutter J, Reich S, Zesiewicz S, Weiner W. Practice parameter: Neuroprotective strategies and alternative therapies for Parkinson disease (an evidence-based review) Report of the Quality Standards Subcommittee of the American Academy of Neurology. Neurology 2006; 66: 976-982.
10) Andreadou E, Nikolaou C, Gournaras F, Rentzos M, Boufidou F, Tsoutsou A, Zournas Ch, Zissimopoulos V, Vassilopoulos D. Serum uric acid levels in patients with Parkinson's disease: Their relationship to treatment and disease duration. Clin Neurol Neurosurg 2010; 111: 724-728.
11) Tuncel D, Inanc Tolun F, Toru I. Serum insulin-like growth factor-1 and nitric oxide levels in Parkinson's disease. Mediators Inflamm 2009; 2009: 132464.

12) Inoue H, Hisamatsu K, Ando K, Ajisaka R, Kumagai N. Determination of nitrotyrosine and related compounds in biological specimens by competitive enzyme immunoassay. Nitric Oxide 2002; 7: 11-17.

13) Marinus J, Visser M, Stiggelbout A, Rabey J, Martinez-Martin P, Bonuccelli U, Kraus PH, Hilten JJ. A short scale for the assessment of motor impairments and disabilities in Parkinson's disease: the SPES/SCOPA. J Neurol Neurosurg Psychiatry 2004; 75: 388-395.

14) Goetz C. The Unified Parkinson's Disease Rating Scale (UPDRS): Status and recommendations. Mov Disord 2003; 18: 738-750.

15) Schrag A, Sampaio C, Counsell N, Poewe W. Minimal clinically important change on the Unified Parkinson's Disease Rating Scale. Mov Disord 2006; 21: 1200-1207.

16) Sanyal J, Bandyopadhyay S, Banerjee T, Mukherjee S, Chakraborty D, Ray B, Rao V. Plasma levels of lipid peroxides in patients with Parkinson's disease. Eur Rev Med Pharmacol Sci 2009; 13: 129-132.

17) Chan M, Mattiacci J. Nitric oxide: actions and roles in arthritis and diabetes. Foot 2001; 11: 45-51.

18) Naseem K. The role of nitric oxide in cardiovascular diseases. Mol Aspects Med 2005; 26: 33-65.

19) Low S. Application of pharmaceuticals to nitric oxide. Mol Aspects Med 2005; 26: 97-138.

20) Larsen T, Nilsen V, Andersen D, Francis G, Rustad P, Mansoor M. Comparison of high-pressure liquid chromatography (HPLC) and Griess reagent-spectroscopic methods for the measurement of nitrate in serum from healthy individuals in the Nordic countries. Clin Biochem 2008; 41: 1474-1481.

21) Giustarini D, Rossi R, Milzani A, Dalle-Donne I. Nitrite and nitrate measurement by Griess reagent in human plasma: Evaluation of interferences and standardization. Methods Enzymol 2008; 440: 361-380.

22) Setsukinai K, Urano Y, Kakinuma K, Majima H, Nagano T. Development of novel fluorescence probes that can reliably detect reactive oxygen species and distinguish specific species. J Biol Chem 2003; 278: 3170-3175.

23) Huang J, Li D, Diao J, Hou J, Yuan J, Zou G. A novel fluorescent method for determination of peroxynitrite using folic acid as a probe. Talanta 2007; 72: 1283-1287.

24) Boll M, Alcaraz-Zubeldia M, Montes S, Rios C. Free copper, ferroxidase and SOD1 activities, lipid peroxidation and NOx content in the CSF. A different marker profile in four neurodegenerative diseases. Neurochem Res 2008; 33: 1717-1723.

25) Shukla R, Rajani M, Srivastava N, Barthwal M, Dikshit M. Nitrite and malondialdehyde content in cerebrospinal fluid of patients with Parkinson's disease. Int J Neurosci 2006; 116: 1391-1402.