

## Oxidative stress and related diseases. Part 2: Parkinson' disease

G. Nikolova<sup>1</sup>, Y. Karamalakova<sup>1</sup>, V. Mancheva<sup>2</sup>, I. Manchev<sup>2</sup>, A. Zheleva<sup>1</sup>, V. Gadjeva<sup>1\*</sup>

<sup>1</sup>Department of Chemistry and Biochemistry, Medical Faculty, Trakia University, 11 Armeiska Str., 6000 Stara Zagora, Bulgaria

<sup>2</sup>Department of Neurology and Psychiatry, Medical Faculty, University Hospital, Trakia University, 11 Armeiska Str., 6000 Stara Zagora, Bulgaria

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Parkinson' disease is a progressive neurodegenerative movement disorder associated with a selective loss of neurons in the midbrain area called the substantia nigra pars compacta. Pharmacological treatment of PD traditionally has centered on administering medications that restore dopaminergic influence in the basal ganglia. During the early stage of disease, Levodopa administration can produce significant improvements in parkinsonian symptoms moreover, the effects of Levodopa, often diminish after only a few years of continuous use, and this drug often is ineffective in the advanced stages of PD. The aim of current research was to evaluate and compare oxidative status in blood of PD patients treated with Madopar, alone and with a combination of Madopar and vitamin C by several analytical techniques. To realize the aim were investigated: 1) levels of ROS products, ascorbate (Asc•) and NO• radicals as real time oxidative stress biomarkers using Electron paramagnetic resonance (EPR) spectroscopy; 2) the oxidative end products of lipids, proteins and DNA, levels of malondialdehyde (MDA) measured by visible spectrophotometry, protein carbonyl content (PCC) and also 8-hydroxy-2'-deoxyguanosine (8-OHdG) determined by ELISA; and 3) erythrocyte SOD activity by visible spectrophotometry in blood of PD patients.

**Keywords:** Oxidative stress, Parkinson' disease, vitamin C.

### INTRODUCTION

Parkinson's disease (PD), also known as idiopathic or primary parkinsonism/*paralysis agitans*/ is a neurodegenerative disorder (ND) of the central nervous system. Pathologically, the disease is characterized by the proteinaceous cytoplasmic inclusions presence known as Lewi bodies. Contemporary treatment is more efficient during the initial stages of the motor disorders mainly, by the treatment with Levodopa and Dopamine agonists. The Levodopa effects often diminish after few years' continuous use, and in the advanced PD stages, the drug is ineffective. More importantly, the Levodopa administration seems not to delay the PD progression. Simple increase of dopaminergic influence in the basal ganglia does not prevent the deterioration of neurons in the *substantia nigra*. Recent efforts, therefore, have been focused on identifying the factors responsible for neuronal degeneration and for developing medications that might be able to delay nigral cell death [1]. Consequently, with the progress of the disease and neuronal loss, Levodopa becomes ineffective, yielding complications such as dyskinesias. Motor symptoms of PD disease result in dopamine-generating cells loss in the *substantia nigra pars compacta*. The reason for this cell death is unknown, but a number of studies [2, 3] consider

the role of free radicals and implicated oxidative stress (OS) that leads to cellular dysfunction and demise.

OS can cause cellular damage and subsequent cell death because the reactive oxygen species (ROS) oxidize vital cellular components [4]. exhibit increased levels of oxidized lipids [5], proteins [6], and DNA [7]. OS occurs when an imbalance is formed between production of ROS and cellular antioxidant defense mechanisms that include removal of O<sub>2</sub>, scavenging of reactive oxygen/nitrogen species or their precursors, inhibition of ROS formation, binding of metal ions needed for the catalysis of ROS generation and up-regulation of endogenous antioxidant defenses. Various antioxidant therapies have been proposed for reducing oxidative stress in neurodegenerative diseases including PD, but the results with regard to the efficacy of the antioxidant treatments remain controversial [8]. Substances that delay, prevent, or remove oxidative damages to a target molecule are considered as antioxidants. One of the vital roles of the classic antioxidant vitamin C is to protect cellular components from free radical damage. Ascorbic acid has been shown to scavenge free radicals directly in the aqueous phases of cells and the circulatory system [9]. The acid has also been proven to protect membrane and other hydrophobic compartments from such damage by regenerating the antioxidant form of vitamin E. Mechanism of vitamin C antioxidant function, myriad of

\* To whom all correspondence should be sent:

E-mail: : vgadjeva@mf.uni-sz.bg

pathologies resulting from its clinical deficiency, and the many health benefits it provides, are reviewed [7].

In the current study, we explored the influence of vitamin C on the oxidant/antioxidant status of PD patients undergoing prolonged therapy with Madopar whose main ingredient is Levodopa. To achieve the aim of the present research the levels of ROS products formed in real time and end products from oxidation of lipids, proteins and nucleic acid in blood samples isolated from PD patients after therapy with Madopar (Levodopa) alone and in combination with vitamin C were examined and compared.

## MATERIALS AND METHODS

All chemicals used in this study were of analytically grade and purchased from Sigma-Aldrich Chemie GmbH (Germany). Spin-traps *N*-tert-butyl- $\alpha$ -phenylnitrone (PBN) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (Carboxy-PTIO.K), were purchased from Sigma Chemical Co, St. Louis, USA. The ELISA kits for PCC and Oxidative DNA Damage (8-OHdG) measurement were purchased from Cell Biolabs Inc., USA. In our study were included 22 patients hospitalized in the Neurological Clinic of the University Hospital, Stara Zagora, Bulgaria. All studied parameters were compared with those of the 20 control healthy individuals. For PD diagnosing were used the national consensus for PD (medical history and pathological criteria in the neurological condition such as tremor, muscular rigidity, bradykinesia and postural instability), which is in compliance with the PD society brain bank clinical diagnostic criteria. The group of selected patients is homogenous according to the clinical features in parallel with the disorders of the cognition, mood, behavior, and mentation, etc. The Unified Parkinson Disease Rating Scale (UPDRS) was used for assessment of the PD and the level of pathological changes was assessed with the modified scale of Hoehn and Yahr. The patient capability for everyday activity was assessed according to the Schwab and England scale.

The PD patients group were treated only with Madopar (250 mg), containing –Levodopa 200 mg and Benserazide 50 mg from 2 to 4 years. Afterwards, the same patients have received both Madopar (250 mg) and vitamin C (1000 mg) for 2 months. Informed consent was obtained from all PD patients and healthy volunteers enrolled in this study, according to the ethical guidelines of the Helsinki Declaration (1964). Fasting samples of venous blood were collected in the morning between 8.00 and 10.00 a.m. Blood for

determination of MDA, NO $\bullet$  and ROS products was collected in tubes containing 10% EDTA (ethylenediaminetetraacetic acid). Whole blood in a covered test tube (without anticoagulant) was collected for determination of PCC, 8-OHdG and erythrocyte SOD activity. All samples from each subject were split and run in triplicate.

### *Ex vivo electron paramagnetic resonance (EPR) study*

EPR measurements were performed at 22 $^{\circ}$ C temperature on an X-band EMX<sup>micro</sup>, spectrometer Bruker, Germany. The experiments were carried out in triplicate and repeated thrice. Spectral processing was performed using Bruker WIN-EPR and *Sinfonia* software.

### *Ex vivo evaluation of the levels of ROS products*

The ROS levels were determined according to [10] with modification. To investigate in real time formation of ROS in the sera of PD patients and controls *ex vivo* EPR spectroscopy combined with PBN as a spin-trapping agent was used.

### *Ex vivo evaluation of the levels of Asc $\bullet$*

Endogenic ascorbic acid can be oxidized by ROS to a stable ascorbate radical and the latter can be detected by direct EPR method which does not interfere with the biochemical processes. The levels of Asc $\bullet$  were studied according to Bailey *et al.* [11] with some modification.

### *Ex vivo evaluation of the levels of $\bullet$ NO radicals*

Based on the methods published by Yoshioka *et al.* [12] and Yokoyama *et al.* [13], we developed and adapted the EPR method for estimation of the levels of  $\bullet$ NO radicals in serum.

### *Determination of SOD activity in erythrocytes*

CuZn-SOD activity was determined as described by Sun *et al.* [14] with minor modifications. Results were expressed as units per g hemoglobin (U/gHb).

### *Determination of lipid-peroxidation products (MDA)*

Total amount of lipid peroxidation in the plasma of healthy volunteers and patients was estimated using the thiobarbituric acid (TBA) method of [15], by measuring the malondialdehyde (MDA) reactive products at 532 nm. Results were expressed in  $\mu$ mol/l.

### *Protein carbonyl content*

PCC was measured by using a commercial ELISA kit following manufacturer's instructions.

Quantity of 8-hydroxy-2'-deoxyguanosine

The measurement of 8-OHdG was carried out using a commercial ELISA kit, following manufacturer's instructions. The kit has an 8-OHdG detection sensitivity range of 100 pg/mL - 20 ng/mL.

RESULTS

The ROS products (Fig.1) in the sera of PD patients treated with Madopar was insignificantly higher compared to controls (mean  $3.47 \pm 1.17$  vs mean  $3.09 \pm 0.41$ ,  $p > 0.05$ , t-test). The ROS products in PD patients treated with a combination of Madopar + vitamin C were less than in the controls, (average  $2.81 \pm 0.3$  versus mean  $3.09 \pm 0.4$ ,  $p > 0.05$  t-test). However, there was a statistically significant difference between PD group treated with the combination of Madopar and vitamin C and PD treated only with Madopar (mean  $2.81 \pm 0.3$  vs. mean  $3.47 \pm 1.17$ ,  $p = 0.001$ , t-test).

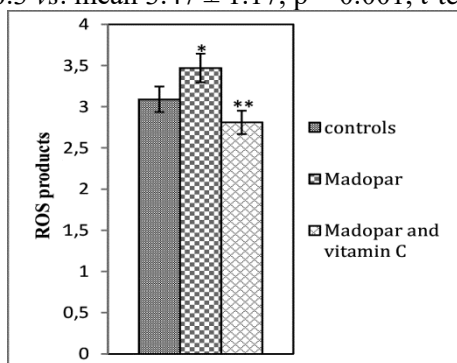


Fig. 1. Levels of ROS products expressed in arbitrary units in sera of controls, PD patients with Madopar therapy and PD patients with combined therapy – Madopar +vitamin C. (\*)  $p < 0.001$  – PD group treated with Madopar vs controls; (\*\*)  $p < 0.001$  –PD group treated only with Madopar vs group treated with Madopar + vitamin C.

Statistically significant increased Asc• levels (Fig. 2) were observed in PD patients treated with Madopar compared to controls (mean  $2.49 \times 10^6 \pm 0.35$ , vs mean  $0.86 \times 10^6 \pm 0.02$ ,  $p < 0.001$ , t-test). Moreover, the Asc• levels in PD patients treated with Madopar +vitamin C did not show statistically significant difference compared to the controls (mean  $0.99 \times 10^6 \pm 0.03$ , vs mean  $0.86 \times 10^6 \pm 0.02$ ,  $p > 0.05$ , t-test), but there was a statistically significant difference comparing to patients treated with Madopar alone (mean  $0.99 \times 10^6 \pm 0.03$ , vs mean  $2.49 \times 10^6 \pm 0.35$ ,  $p = 0.001$ , t-test).

Statistically significant higher NO• levels (Fig. 3) were measured in Madopar-treated patients compared to controls (mean  $4.85 \pm 0.4$ , vs mean  $26.74 \pm 1.2$ ,  $p=0.00$ , t-test). It should be noted that

after treatment with Madopar + vitamin C, statistically significantly lower NO• levels were found compared to those measured in the same patients prior to inclusion of vitamin C in the therapy (mean  $26.74 \pm 1.2$ , vs mean  $14.86 \pm 0.4$ ,  $p=0.00$ , t-test).

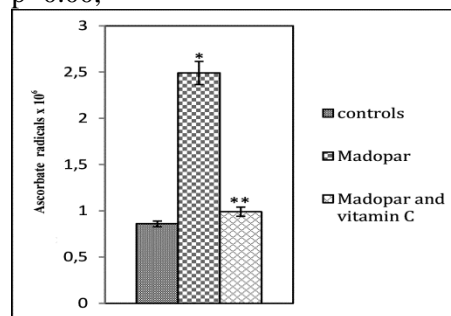


Fig. 2. Levels of Asc radicals expressed in arb. units in sera of controls, PD patients with Madopar therapy and PD patients with combined therapy –Madopar + vitamin C. (\*)  $p < 0.001$  – PD group treated with Madopar vs controls; (\*\*)  $p < 0.001$  – Madopar treated PD group vs PD group treated with Madopar + vitamin C.

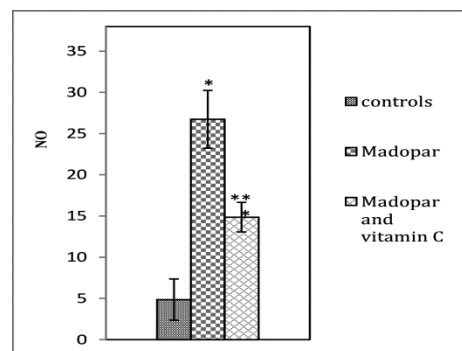
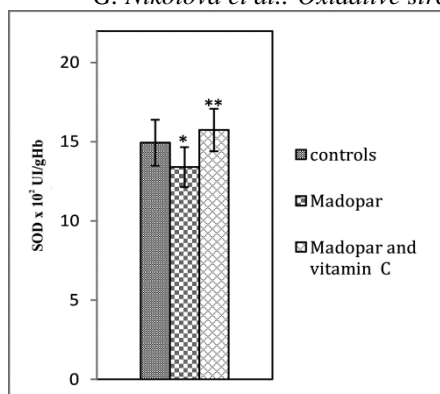


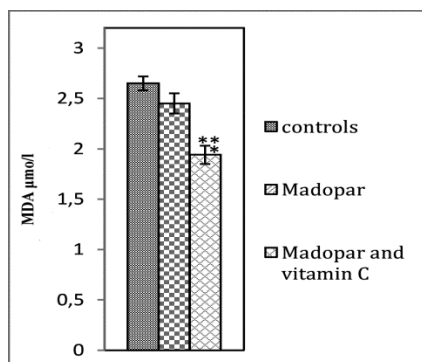
Fig. 3. Levels of NO radicals expressed in arb. units in sera of controls, PD patients with Madopar therapy and PD patients with combined therapy –Madopar and vitamin C. (\*)  $p < 0.001$  – PD group treated with Madopar vs controls; (\*\*)  $p < 0.001$  –PD group treated only with Madopar vs PD group treated with Madopar + vitamin C.

SOD activity (Fig. 4) in PD group treated with Madopar was statistically lower compared to controls (mean  $13.4 \times 10^2 \pm 1.34$  U/gHb vs  $14.94 \times 10^2 \pm 1.45$  U/gHb,  $p < 0.05$ , Student's t-test), as well as compared to the SOD measured in the same patients after inclusion of vitamin C (mean  $13.4 \times 10^2 \pm 1.34$  U/gHb vs  $15.75 \times 10^2 \pm 1.57$  U/gHb). SOD in PD patients measured after inclusion of vitamin C was close to that of the controls (mean  $15.75 \times 10^2 \pm 1.57$  U/gHb vs  $14.94 \times 10^2 \pm 1.45$  U/gHb,  $p > 0.05$ , Student's t-test).



**Fig. 4.** SOD activity in controls, PD patients with Madopar therapy and PD patients with combined therapy –Madopar +vitamin C. (\*)  $p < 0.0001$  PD group treated only with Madopar vs controls; (\*\*)  $p < 0.0001$  - Madopar treated PD group vs PD group treated with combination of Madopar + vitamin C.

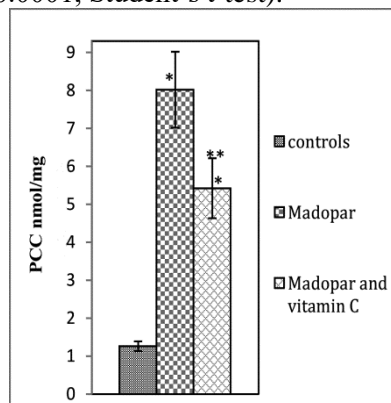
MDA levels (Fig. 5) measured in plasma of PD patients treated with Madopar alone was lower than the controls (mean  $2.45 \mu\text{mol/l} \pm 0.09$  vs mean  $2.65 \mu\text{mol/l} \pm 0.07$ ,  $p > 0.05$ , Student's t-test). MDA measured in PD patients treated with Madopar + vitamin C compared to controls (mean  $1.94 \mu\text{mol/l} \pm 0.07$ , vs  $2.65 \pm 0.07 \mu\text{mol/l}$   $p < 0.0001$ ) and to PD patients treated only with Madopar (mean  $1.94 \mu\text{mol/l} \pm 0.07$ , vs  $2.45 \mu\text{mol/l} \pm 0.09$ ,  $p < 0.001$ , Student's t-test, respectively) were significantly reduced.



**Fig. 5.** MDA plasma levels in controls, PD patients with Madopar therapy and PD patients with combined therapy –Madopar + vitamin C. (\*)  $p < 0.0001$  - PD group treated with Madopar + vitamin C. (\*\*)  $p < 0.0001$  - PD group treated with combination Madopar + vitamin C vs Madopar treated PD group.

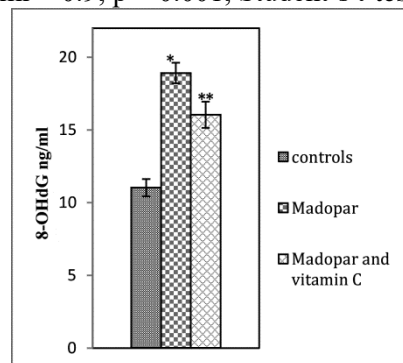
PD patients treated with Madopar showed statistically higher PCC (Fig. 6) compared to controls (mean  $8.02 \text{ nmol/mg} \pm 0.7$ , vs  $1.26 \text{ nmol/mg} \pm 0.13$ ,  $p = 0.0001$ , Student's t-test). Statistically significant difference was also measured in PD patients treated with Madopar +vitamin C compared to control (mean  $5.42 \text{ nmol/mg} \pm 0.43$ , vs  $1.26 \text{ nmol/mg} \pm 0.13$ ,  $p = 0.001$ , Student's t-test). The patients, undergoing

combined therapy, expressed significantly lower PCC levels in comparison with Madopar treated (mean  $5.42 \text{ nmol/mg} \pm 0.43$ , vs  $8.02 \text{ nmol/mg} \pm 0.13$ ,  $p = 0.0001$ , Student's t-test).



**Fig. 6.** PCC in controls, PD patients with Madopar therapy and PD patients with combined therapy – Madopar + vitamin C. (\*)  $p < 0.001$ –vs controls; (\*\*)  $p < 0.0001$  Madopar treated PD group vs PD group treated with combination of Madopar + vitamin C.

Significant increase of 8-OHdG (Fig.7.) levels was found in PD patients treated with Madopar, compared to the controls (mean  $18.91 \text{ ng/ml} \pm 0.9$  vs  $11.03 \text{ ng/ml} \pm 0.6$ ,  $p < 0.0001$ , Student's t-test). PD patients treated with Madopar + vitamin C were in contrast compared to controls (mean  $16.04 \text{ ng/ml} \pm 0.8$  vs  $11.03 \text{ ng/ml} \pm 0.6$ ,  $p < 0.001$ , Student's t-test). The amount of 8-OHdG measured in PD patients treated with Madopar + vitamin C was significantly reduced in comparison to Madopar treated PD patients (mean  $16.04 \text{ ng/ml} \pm 0.8$  vs  $18.91 \text{ ng/ml} \pm 0.9$ ,  $p < 0.001$ , Student's t-test).



**Fig. 7.** DNA damage, measured as 8-OHdG (ng/ml) in controls, PD patients with Madopar therapy and PD patients with combined therapy –Madopar and vitamin C. (\*)  $p < 0.001$ –vs controls; (\*\*)  $p < 0.0001$  Madopar treated PD group vs PD group treated with combination of Madopar + vitamin C.

## DISCUSSION

Investments in drug research and development have increased in recent decades, but the annual number of truly innovative new medicines has not increased accordingly, which creates a problem in

the pharmaceutical industry to replace the loss of revenues due to patent expiration [16]. Therapy with Madopar is still the most efficient method of PD treatment [17]. Despite evidence that OS has played a significant role in PD, a good target for pharmacological management has to be determined. ROS should be a therapeutic target in PD, this is necessary to recognize that an antioxidant in chemical systems may be not an efficient agent in biological ones. The effectiveness of antioxidants is probably limited by their bioavailability and the fact that they would have to be present in high concentrations to be able to compete with endogenous targets. Therefore, the current study was undertaken to clarify the protective effect of vitamin C against oxidative toxicity of Madopar in PD patients. The most susceptible biological macromolecules to oxidative injuries are membrane lipid, proteins, and DNA. Moreover, in biosystems quantification of final products of oxidative damage constitutes indirect evidence of OS as a result of increased generation of ROS and RNS. The above mentioned facts prompted us using proper techniques to explore selected OS parameters such as Asc•, ROS products, NO•, SOD activity and levels of final products of lipids, proteins and DNA oxidation in blood of PD patients subjected to prolonged treatment with Madopar and to evaluate the effect of vitamin C on the levels of these parameters after inclusion in the therapy of the same PD patients. In the course of lipid peroxidation a variety of unstable radical species are formed that can be measured [18]. Short-lived radicals such as ROS formed during *in vivo* oxidation can be proved only by EPR spectroscopy using proper spin traps or spin probes [19,20]. By using spin probe CMH (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine) a quantitative EPR method was developed to monitor ROS production in physiological and pathological conditions. It was found that increased ROS production corresponded to increased plasma TBARS and protein carbonyl concentrations in patients with neurodegenerative disorders such as Mild Cognitive Impairment, and Amyotrophic Lateral Sclerosis, comparing to controls [20]. All changes indicated increased OS are directly related to an increase in ROS production [20]. The higher ROS levels in Madopar treated patients in comparison with controls means that oxidative processes [21] are available in real time. At present, Asc• is used as an endogenous marker for OS in biological systems. From the thermodynamic point of view, the ascorbate ranks at the end of oxidative radicals series. Obviously, oxidative species as hydroxyl (•OH); alkylperoxyl (ROO•), lipidperoxyl

(LOO•), tocoperoxyl (•TO) radicals and peroxynitrite (ONOO<sup>-</sup>) have a higher redox potential and can be reduced, thereby generating Asc• radical [22]. In biological systems the endogenous ascorbic acid, oxidized by ROS to stable Asc• radical possessing comparatively long half-life, which in aqueous solution and at room temperature can be readily detected [23]. Significantly increased Asc• levels after prolonged Madopar treatment compared to controls indicate the presence of ongoing oxidative processes in PD patients. Considering the above-mentioned thermodynamic order of oxidative species, it is obvious that the four-fold higher levels of Asc• found in the PD patients *versus* the controls was a result of increased OS due to increased generation of oxidative species having a higher redox potential than the ascorbate. The consideration was supported by the higher levels of ROS products and NO• measured in the patient's group compared to controls.

The increase in the OS due to the low activity of antioxidant enzymes might cause many secondary complications and may contribute to the neurodegeneration in PD [24]. SOD is a scavenging enzyme and is considered as the first line defense against ROS overproducing. Various research groups have investigated OS in blood cells of PD patients and reported controversial results related to erythrocyte activities of SOD, CAT and possible correlation with age, duration and stage of PD [25, 26]. The erythrocyte SOD was decreased only in treated patients and non-treated PD patients [27]. Our study demonstrated a significant decrease in erythrocyte SOD activity in Madopar treated PD patients that corresponds to the increased levels of ROS in the same group. Moreover, in PD patients was also established increase in the NO• levels. The administration of therapeutic agents results in a greater degree of OS than that induced by the disease itself [28-30]. The prolonged drug treatment could lead to further OS and imbalance between production and elimination of ROS, and could contribute to different complications during the course of the PD [16]. Further, to explore how the increased production of ROS and NO reflects on the OS in PD patients subjected to prolonged treatment with Madopar we studied end products of lipids, proteins and DNA oxidation. Well known is that oxidative modification of proteins causes in the greatest extent formation of protein carbonyl groups, therefore the level of the latter has become the most commonly used marker for protein oxidation during OS, aging and neurodegenerative diseases [31]. In fact, DNA also cannot escape oxidative attacks and typical example is

deoxyguanosine that is converted by ROS to 8-hydroxydeoxyguanosine (8-OHdG), a biomarker of oxidative DNA damage [32]. MDA level in plasma of PD patients was insignificantly lower than that of the controls. Lots of studies reported that at early stages of Parkinson, plasma MDA level peaked, while at the late stages of the disease this level even fell below the controls [33, 34], and established negative correlation between plasma lipid peroxidation and intake of levodopa dose. Formerly, evidence was provided that levodopa treatment may cause decrease in plasma lipid peroxidation. Current result provides additional evidence that prolonged Madopar (levodopa) treatment may decrease plasma lipid peroxidation. Another indirect proof for involvement of ROS in drug-induced toxicity is the overcome of the OS by adding typical antioxidants [17, 35]. It is known that antioxidants are needed to prevent the formation and oppose the actions of free radicals. There is evidence that a higher intake of vitamin C and other antioxidants is associated with reduced risk of degenerative or chronic diseases, probably through antioxidant mechanisms [29,36]. Improved antioxidant status helps to minimize oxidative damage, and thus can delay or prevent pathological changes. This suggests the possible utility of antioxidant-based dietary strategies for lowering the risk of chronic age-related, free radical-induced diseases, and their complications. Vitamin C is the first line of the non -enzymatic antioxidant defense against oxygen radicals [37]. Overproduction of free radicals accompanies all neurodegenerative diseases, and ascorbic acid may block some processes associated with ROS overproduction [38]. Water-soluble vitamin C acts as a chain-breaking antioxidant and in biological systems scavenges free radicals such as ROS by donating electrons and thus may prevent other biological molecules from being oxidized [36]. To check the possibility ROS to be involved in drug-induced oxidative toxicity vitamin C was added to Madopar therapy of the same patients at a dose of 1000 mg per day for 2 months. We assumed that the significant decline in ROS levels and bringing them close to the control group after inclusion of vitamin C was due to the classic antioxidant that largely overcomes the ongoing oxidative processes in PD patients subjected to prolonged treatment with Madopar. This assumption was additionally confirmed by the significant decrease in PCC and 8-OHdG sera levels measured in PD patients after inclusion of vitamin C in their therapy. It should be emphasized that vitamin C in the tested dosage and duration of administration successfully overcomes

lipid oxidative toxicity induced by both Levodopa long-term treatment and the disease, itself.

## CONCLUSION

Regardless of a lot of clinical studies. suitable antioxidants by which effectively to treat neurodegenerative diseases including PD are not yet found. We consider that taken together, recently published results of the present and other authors, as well as the present survey, launched a promising opportunity and a new direction in search of relationship between different oxidative stress biomarkers in brain tissue and blood of PD patients, which further might help in developing efficient antioxidant schemes to find application in treatment of PD.

**Conflict of interests:** No conflict of interests to declare.

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## ОКСИДАТИВЕН СТРЕС И СВЪРЗАНИ С НЕГО БОЛЕСТИ. ЧАСТ 2. БОЛЕСТ НА ПАРКИНСОН

Г. Николова<sup>1</sup>, Я. Карамалакова<sup>1</sup>, В. Манчева<sup>2</sup>, И. Манчев<sup>2</sup>, А. Желева, В. Гаджева<sup>1\*</sup>

<sup>1</sup> Катедра по химия и биохимия, Медицински факултет, Тракийски университет, ул. Армейска 11, 6000 Стара Загора, България

<sup>2</sup> Катедра по неврология и психиатрия, Медицински факултет, Университетска болница, Тракийски университет, ул. Армейска 11, 6000 Стара Загора, България

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(Резюме)

Болезтта на Паркинсон (PD) е прогресивно невродегенеративно разстройство на движението, свързано със селективна загуба на неврони в областта на средния мозък, наречена *substantia nigra pars compacta*. Фармакологичното лечение на PD традиционно се състои в прилагане на лекарства, възстановяващи допаминергичното влияние в базалните ганглии. През време на началния етап на болестта, прилагането на Levodopa може да доведе до значително подобрене на симптомите на Паркинсоновата болест, но влиянието на Levodopa често отслабва след няколко години непрекъсната употреба и това лекарство често е неефективно в напредналите стадии на PD. Целта на настоящото изследване е да се оцени и сравни оксидативният статус в кръвта на пациенти с PD, третирани само с Madopar, и с комбинация от Madopar и витамин С с използване на различни аналитични техники. За целта са изследвани: 1) нивата на продуктите на реактивни кислородни видове ROS, аскорбатни (Asc•) и NO• радикали като биомаркери на оксидативния стрес в реално време с използване на EPR спектроскопия; 2) оксидативните крайни продукти на липиди, протеини и DNA, нивата на малондиалдехид (MDA), измерени чрез спектрофотометрия във видимата област, съдържанието на протеинов карбонил (PCC), а също и на 8-хидрокси-2'-деоксигуанозин (8-OHdG), определени с ELISA тест; 3) еритроцитната SOD активност чрез спектрофотометрия във видимата област на кръв от пациенти с PD.