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Antioxidant status and clinicopathologic parameters in patients with Parkinson's disease

Antioksidativni status i kliničko-patološki parametri kod obolelih od Parkinsonove bolesti

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Abstract

Backgroun/Aim. Constant production of free radicals and antioxidants (AO) in cells is a part of normal cellular function. Their imbalance might take a part in pathophysiology of many diseases, including Parkinson's disease (PD). Evaluation of the disease status, prooxidant-antioxidant balance (PAB) and antioxidants are being widely estimated. The aim of this study was to examine potential interaction between several AO variables: glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and PAB, and clinicopathologic features of patients with PD, particularly the Hoehn and Yahr (H&Y) stage. **Methods**. A multivariate analysis of variance (MANOVA) was conducted to analyze mean differences between clinicopathologic characteristics (gender, age at examination, duration of the disease, and the H&Y stage) and AO variables of PD patients and those of age/sex matched healthy controls. The study included 91 patients with idiopatic PD patients and 20 healthy persons. **Results.** The multivariate effect size was estimated at 0.269 ($p < 0.001$), implying that 27.0% of the variance of the dependent variables was accounted for the H&Y stage. Univariate tests showed that there were significant differences ($p < 0.001$) across the H&Y stage of all AO variables. The H&Y stage remained significant predictor after controlling for the second variable, the disease duration ($p < 0.001$, $\eta^2 = 0.249$), and there were still significant differences across the H&Y stage of all variables, with effect size (η^2) ranging from 0.132 ($p = 0.011$) (lnGSH) to the still high values of 0.535 (lnPAB), 0.627 (lnSOD) and 0.964 (lnCAT). **Conclusion**. The results indicate that higher level of oxidative stress in blood of PD patients is possibly related to the PD stage. Along with reduction of SOD and GSH levels, CAT activity was elevated in comparison to these values in healthy subjects. Furthermore, PAB was shifted toward oxidative stress.

Key words:
parkinson disease; disease progression; free radicals; antioxidants; demography.

Apstrakt

Uvod/Cilj. Ćelijska homeostaza zasniva se na konstantnoj produkciji slobodnih radikala i antioksidanasa (AO). Svako narušavanje njihove ravnoteže može dovesti ili učestvovati u patofiziološkim promenama mnogih bolesti, uključujući i Parkinsonovu bolest (PB). Kako bi se pratio status bolesti, koristi se veliki broj parametara, uključujući i prooksidativni-antioksidativni balans (PAB) i AO, koji ujedno predstavljaju i fokus ispitivanja ove studije. Stoga, cilj ove studije je bilo ispitivanje potencijalne interakcije između AO varijabli: glutation (GSH), superoksid dismutaza (SOD), katalaza (CAT) i PAB i kliničko-patoloških osobina PB bolesnika, najviše Hoehn i Yahr (H&Y) stepena bolesti. **Metode.** Multivarijantna analiza varijanse (MANOVA) korišćena je za analizu međusobnih razlika između kliničko-patoloških karakteristika (pola, starosti, dužine trajanja bolesti i H&Y stepena bolesti) i AO varijabli bolesnika sa PD sa onima od zdravih osoba. Studija je uključila ukupno 111 ispitanika, 91 bolesnika kojima je dijagnostifikovana idiopatska PB i 20 zdravih osoba. **Rezultati.** Multivarijantni efekat je bio procenjen na 0,269 ($p < 0,000$), što implicira da se 27,0% varijanse za-

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visne varijable odnosi na H&Y stepen bolesti. Univarijantni test je pokazao da postoji statistički značajna razlika ($p < 0,001$) kroz H&Y stepen bolesti svih AO varijabli. H&Y stepen bolesti ostao je značajan predikator i nakon uvođenja druge varijable, dužine trajanja bolesti ($p < 0,001$; $\eta^2 = 0,249$). Pokazano je da je ostala značajna razlika kroz H&Y stepen bolesti za sve varijable, tako da se jačina odnosa dve varijable kretala od 0,132 (lnGSH) do i dalje visokih vrednosti: 0,535 (lnPAB), 0,627 (lnSOD) i 0,964 (lnCAT). **Zaključak.** Rezultati pokazuju da je visoki nivo oksidativnog stresa u krvi obolelih od PB verovatno povezan sa stepenom bolesti. Zajedno sa smanjenjem aktivnosti SOD i nivoa GSH, aktivnost CAT se povećava u poređenju sa ovim vrednostima kod zdravih osoba. Pored toga, PAB ukazuje na povećani oksidativni stres kod obolelih od PB.

Ključne reči:
parkinsonova bolest; bolest, progresija; slobodni radikali; antioksidansi; demografija.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease, histologically characterized by progressive loss of dopaminergic neurons in *substantia nigra pars compacta* (*SNpc*) and formation of Lewy bodies [1]. It is manifested by cardinal features such as bradykinesia, rigidity, tremor and postural instability, and good response to levodopa (L-dopa) is often used to support the diagnosis of PD [2]. Although the exact mechanism of PD pathogenesis still remains unclear, studies have indicated that oxidative stress (OS), inflammation, mitochondrial dysfunction and proteasomal inhibition are the major factors that accelerate dopaminergic neurodegeneration [3].

Oxidative stress is defined as an imbalance between the production of reactive oxygen species (ROS) and antioxidant (AO) defense capacity. ROS are generally short-lived and highly reactive molecules derived from oxygen [4], varying in their site of formation, physiological function, reactivity and biological half-life. They include free radicals, such as hydroxyl and superoxide radicals, and non-radicals including hydrogen peroxide (H_2O_2) and singlet oxygen [5]. Maintenance of the physiological level of ROS is basically regulated by antioxidant enzymes (AOE) and small antioxidant molecules [6].

Antioxidant enzymes include superoxide dismutases (SODs), catalase (CAT), glutathione peroxidases (GPxs), glutathione reductases (GRs) and glutathione-S-transferases (GSTs), while non-enzymatic antioxidants are represented by glutathione (GSH), ascorbic acid (vitamin C), α-tocopherol (vitamin E), flavonoids, etc. [7]. The main function of SOD is catalyzing the breakdown of highly reactive superoxide anion into oxygen and the less reactive H_2O_2, which is further decomposed to water and oxygen by CAT or GPx [8]. Disturbance of AOE activity is strongly implicated in a variety of age–related brain disorders [9].

Glutathione is the major small AO molecule [6], with the concentration of 1–3 mM in the brain cells [10]. It is highly abundant in the cytosol (1–11mM), nucleus (3–15 mM) and mitochondria (5–11 mM) [11]. In some studies, a much lower concentration of 2 µM was found in blood plasma [10]. GSH can reduce superoxide radicals, hydroxyl radicals, and peroxynitrites, reacting alone or with other enzymes, such as GPx or GST [12].

Other than individual molecules, one of the important parameters for oxidative stress evaluation is a prooxidant-antioxidant balance (PAB), which determines a state of dynamic balance between free radicals that are produced and those utilized (scavenged) [13].

Similar to other diseases, a disturbed AO balance renders PD patients more vulnerable to OS. Thus, to further evaluate its degree, the present study investigated PAB and AO enzymes (SOD, CAT), as the first line of defense against ROS, and GSH level in the blood of PD patients, compared to healthy subjects. Furthermore, the relation of AO parameters with clinicopathologic features of PD patients such as gender, age, duration of the disease, and the Hoehn and Yahr (H&Y) staging was estimated.

Methods

Participants

The study comprised 91 patients with idiopathic PD, and 20 healthy controls, originated in the Republic of Serbia. All blood samples were collected at the Neurology Clinic, Clinical Center of Serbia in Belgrade. The study was performed in compliance with the ethical principles of the Declaration of Helsinki and all applicable national laws and regulations. The study protocol was approved by the Ethics Committee of the Clinical Centre of Serbia, Belgrade, and written informed consent was obtained from each patient prior to study engagement. All patients had idiopathic PD diagnosed in accordance with UK brain bank criteria [14]. Inclusion criteria were disease duration (up to 25 years), age (30–75 years), the Hoehn and Yahr (H&Y) stage (I–IV), receiving symptomatic PD therapy and a stable dose of L-dopa for longer than 3 months. Patients with current evidence of a recent diagnosis of malignancy, marked autonomic disturbances, a renal insufficiency or failure, hepatitis, serious and/or unstable gastrointestinal, hematologic or other medical disorders, as well as subjects using antipsychotics were excluded from the study. The clinicopathologic features of patients including age, gender, disease duration and the H&Y stage of the disease is given in Table 1.

Blood sampling and biochemical measurements

Venous blood samples were collected from each patient using conventional techniques into Vacutainer (BD Diagnostics, Plymouth, UK) tubes with K_2EDTA as an anticoagulant. For PAB measurement, one batch was centrifuged at 1,500 g, for 10 min, at 4°C, within 30 min of collection. Plasma was carefully separated and stored at -80°C until further processing.

Table 1

Demographic and clinical data of patients with Parkinson's disease PD

Characteristic	Values
Gender, n (%)	
male	60 (65.9)
female	31 (34.1)
Age at examination (years), mean ± SD	62.7 ± 9.7
< 59	28 (30.8)
59–70	44 (48.3)
> 70	19 (20.9)
Age at disease onset (years), mean ± SD	53.8 ± 9.1
Disease duration (years), mean ± SD	8.8 ± 6.2
< 3	18 (19.8)
3–8	35 (38.5)
> 8	38 (41.8)
H&Y stage, n (%)	
1	9 (9.9)
2	31 (34.1)
3	27 (29.7)
4	24 (26.4)

H&Y – Hoehn and Yahr.

For enzyme activity measurements, the second batch of unfrozen blood was used. All blood samples were diluted with cold dH_20 1:3 (v/v), vortexed and centrifuged for 1 min (10,000 g, 15 min, 4 °C). Supernatants were collected and kept at -80 °C till the assay.

For GSH measurement the blood was prepared as recommended by the kit producer (BIOXYTECH® GSH-420™, OXIS International Inc., Foster City, CA, USA).

Assays

Total SOD activity was measured using Superoxide Dismutase Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA). The reaction between superoxide radicals (O_2^-) and tetrazolium salt, generated by xanthine oxidase, results in the development of formazan dye, with max absorbance on 450 nm. SOD inhibits this reaction by dismutation of $O_2^{\cdot-}$ and one unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of superoxide radical. Measurements were performed in a microplate reader (Wallac 1420 Victor [2], Perkin Elmer Inc., Waltham, MA, USA).

Total GSH concentration was determined by the BIOXYTECH® GSH-420™ Assay (OXIS International, Inc., Foster City, CA, USA). The measurement of total GSH concentration was performed in three colorimetric reaction steps. Tris (2-carboxyethyl) phosphine (TCEP) as a reducing agent, reduces all oxidized glutathione present in the sample. During the second step, chromogen (4-chloro-1-methyl-7-trifluoromethyl-quinolinium methylsulfate) reacts with thiols in the sample and forms thioethers. Addition of base (NaOH) raises reaction mixture pH over 13 and chromophoric thione is formed as a result of β–elimination specific to the GSH-thioether. GSH concentration is directly proportional to the absorbance at 420 nm.

Catalase activity measurement was performed according to the method by Beutler [15]. The reaction mixture was prepared from 50 µL of a Tris-HCl buffer (1 M Tris-HCl, 5 mM EDTA, pH 8.0), 900 µL of a substrate (10 mM H_2O_2), 30 µL of dH_20, and 20 µL of the sample. Decomposition of H_2O_2 was monitored spectrophotometrically (UV Line 9400, SI Analytics GmbH, Mainz, Germany) at 230 nm, 3 min at 37 °C. One unit of CAT activity is defined as the amount of the enzyme which degrades 1 µmoL of H_2O_2 per min under the assay conditions. The extinction coefficient for H_2O_2 is 0.071 $mM^{-1}cm^{-1}$.

Prooxidant-antioxidant balance

Evaluation of PAB was performed as described previously [16]. Following the incubation for 2 min at room temperature in dark, 200 µL of working solution (1 mL TMB cation solution with 10 mL TMB solution) was added to a 96-well microtiter plate and mixed with 10 µL of plasma sample, standard or blank (dH_2O). The mixture was incubated in a dark place for 12 min, at 37 °C and the reaction was stopped by adding 100 µL of 2 N HCl. The values of PAB in plasma samples were determined at 450 nm, with a reference wavelength of 620 or 570 nm, by comparing optical density (OD) of a sample to the standard curve. PAB values are expressed in arbitrary units (HK).

Statistical analysis

The statistical analyses were performed by the GraphPad Prism and SPSS 18.0 for Windows (SPSS Inc., Chicago, IL, USA). Data are expressed as mean ± SD. General linear model (GLM) was used to test the differences between AO and clinicopathological variables, followed by Dunnett and Scheffe *post hoc* tests. Since examined variables had not passed the normality of the distribution (Shapiro-Wilks test), data were previously log-transformed. Pearson's correlation analysis was performed to test the correlation between AO/clinicopathological variables. The p-value < 0.05 was considered statistically significant.

Results

The average age of healthy controls was 57.5 ± 8.5 years, and for PD patients it was 62.7 ± 9.7 years, with a predominance of males (65.9%). The H&Y stage 1 was the least present (in 9.9% of the patients) (Table 1). The activity of AO enzymes (SOD, CAT), the GSH level and PAB are shown in Figure 1.

A multivariate analysis of variance (MANOVA) was conducted to test mean differences between the H&Y stage and AO variables. Prior to conducting the analysis, the Pearson's correlation was performed between the dependent variables in order to test the correlation assumption (Table 2) and significant pattern of correlations was observed amongst all of the dependent variables. Since the Box's M value of 110.06 ($p < 0.001$) indicated significant difference between the covariance matrices, the Pillais' Trace test was used. The MANOVA effect (Pillais' Trace = 1.07, F = 9.103; $p < 0.001$) showed significant differences among the H&Y stage groups on the linear combination of the dependent variables. The multivariate effect size was estimated at 0.269, implying that 27.0% of the variance of the examined AO parameters was accounted for the H&Y stage.

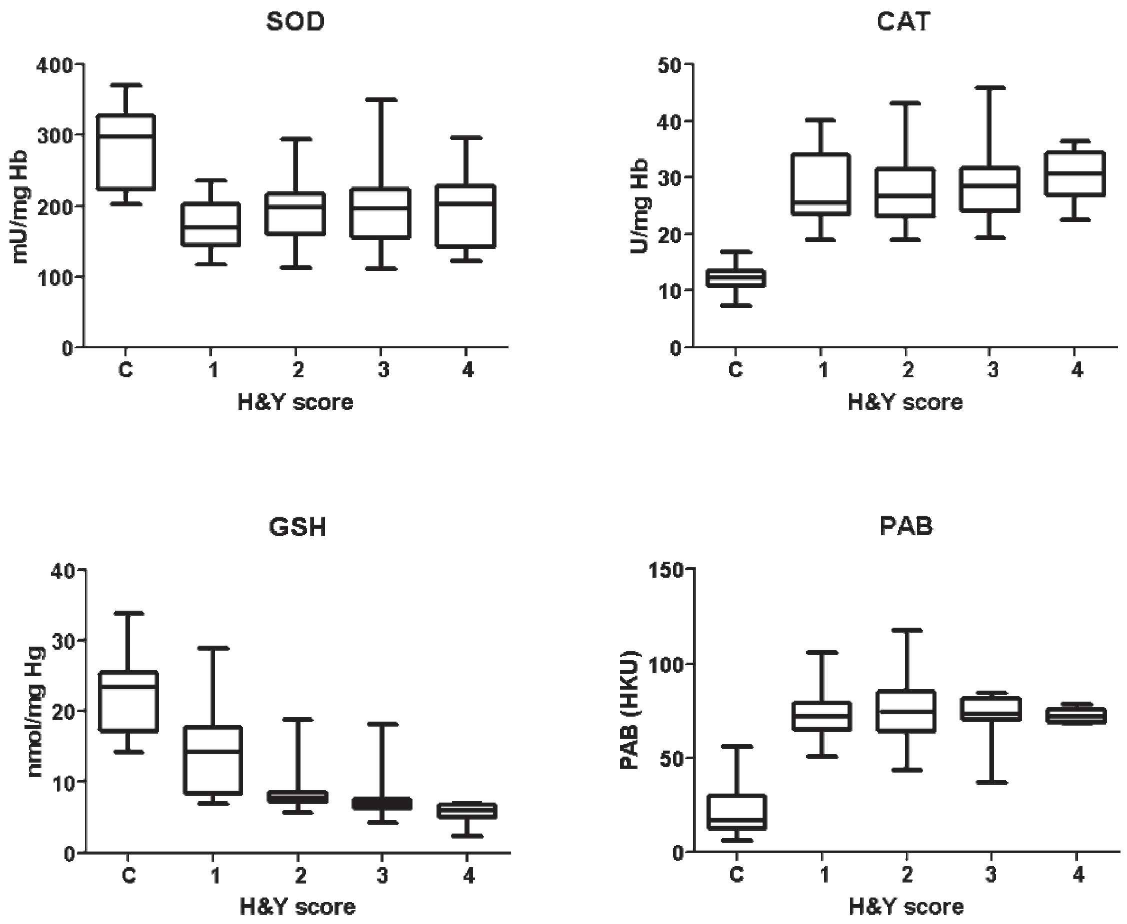

Fig. 1 – Superoxide dismutase (SOD) and catalase (CAT) activity, glutathione concentration (GSH), and prooxidant-antioxidant balance (PAB) in the blood of healthy controls (C) and patients with Parkinson disease of different Hoehn and Yahr (H&Y) scores. Boxes represent values between 25th and 75th percentiles. Medians are given inside the boxes; Whiskers extend between min. and max. values.

Table 2

Pearson's correlation between antioxidant (AO) parameters

Parameters	Pearson's correlation coefficient			
	lnGSH	lnSOD	lnCAT	lnPAB
lnGSH	1	-0.498	0.581	0.595
p (2-tailed)		0.000	0.000	0.000
n	111	111	111	111
lnSOD		1	-0.922	-0.793
p (2-tailed)			0.000	0.000
n		111	111	111
lnCAT			1	0.864
p (2-tailed)				0.000
n			111	111

GSH – glutathione; SOD – superoxide dismutase; CAT – catalase; PAB – prooxidant/antioxidant.

The homogeneity of variance assumption was tested for the AO variables and two (lnGSH and lnPAB) of the four Levene's F tests were statistically significant ($p < 0.05$). Prior to conducting a series of follow-up ANOVAs, the Bonferroni procedure was used to protect against Type I error, adjusting the alpha level to $p < 0.001$. Univariate tests showed that there were significant differences ($p < 0.001$) across the H&Y stage on all AO variables, with effect size (η^2) ranging from 0.365 (lnGSH) to the extremely high values of 0.744 (lnPAB), 0.861 (lnSOD) and 0.988 (lnCAT).

Finally, the series of post-hoc analyses (Dunnett and Scheffe test) were performed to examine individual mean difference comparisons across all H&Y stages and all four AO variables. The results revealed that high effect size observed by univariate analysis was the consequence of the mean differences in AO values between H&Y stages and control values (Dunnett test, $p < 0.001$). Scheffe test did not reveal a significant mean difference in AO values among any of H&Y stages.

In the next step, to test whether H&Y stage remained significant after controlling for the next clinical variable, the disease duration was added as a covariate to the model. The MANCOVA analysis of the effect of the H&Y stage on all AO parameters was still significant (Pillais' Trace = 0.998, F = 7.560; $p <$ 0.000), η^2 0.249. Univariate tests showed that there were still significant differences across the H&Y stage of all AO variables with effect size (η^2) ranging from 0.132 ($p = 0.011$) (lnGSH) to the still high values of 0.535 (lnPAB), 0.627 (lnSOD) and 0.964 (lnCAT) (Table 3a).

There was no significant association between AO parameters and gender (Pillais' Trace = 0.033; F = 0.713; $p = 0.585$; $\eta^2 = 0.033$) or age (Pillais' Trace = 0.70; F = 1.558, $p = 0 .193$; $\eta^2 = 0.070$).

Table 3

General linear model (GLM) analysis of the associations between: a) H&Y stage and antioxidant AO parameters; b) H&Y stage and AO parameters after controlling for disease duration

GLM analisys		lnGSH	lnSOD	lnCAT	lnPAB
a)	F	3.462	38.268	607.374	26.187
	p	0.011	*p* < 0.001	*p* < 0.001	*p* < 0.001
	eta^2	13.2%	62.7%	96.4%	53.5%
b)	F	0.042	3.523	0.650	0.790
	p	0.837	0.064	0.422	0.377
	eta^2	0.000	3.7%	0.7%	0.9%

H&Y – Hoehn and Yahr; GSH – glutathione, SOD – superoxide dismutase, CAT – catalase, PAB – prooxidant/antioxidant balance, eta – quantified variance component.

Discussion

Oxidative stress has long been implicated in pathophysiological mechanisms underlying various neurodegenerative diseases, including PD. Investigation of different oxidant/AO parameters have yielded inconsistent results and it is still challenging to assess these parameters in peripheral blood of patients with PD. The current study is focused on the association of specific AO variables (GSH, SOD, CAT, and PAB) and clinicopathologic features of patients with PD, particularly H&Y stage.

Among all ROS-scavenging enzymes, SOD is often regarded as the first line of defense and there is sufficient evidence relating superoxide anion to human diseases, such as PD [17]. The results of our study showed decreased SOD activities in PD patients compared to healthy subjects, which is in accordance with the findings of some authors [18–21] while the others [22–25] reported increased SOD activity or no significant change at all [26, 27]. It is known that AO enzymes are regulated through the AO system to cope with acute or mild OS; however, severe or prolonged OS may induce consumption and decrease of enzyme activity. The decrease of SOD observed in our study might involve inactivation of SOD by ROS or some posttranslational modifications [28]. This observation is comparable with the fact that reduced activity of blood SOD is detected in many chronic diseases such as obstructive pulmonary disease [29], renal failure [30], as well as in some neurological disorders [31]. Chronic OS has already been speculated to cause antioxidant consumption and thus a decline in antioxidant levels [32]. Another possible reason for decreased SOD level could be in mutations that not only provoke a decline in its activity but also induce self-aggregation of mutated SOD proteins – an initial cause of neuron malfunction leading to the disease, as already shown in a cell culture model of amyotrophic lateral sclerosis [33]. The confirmation of such assumptions requires more extensive research in the field of molecular events related to this disease.

The term OS describes the condition where free radicals production exceeds a capacity of AO system. Studies indicated different findings of erythrocyte CAT activity in PD patients in which no significant changes [27, 34] or deficit [18, 21] of CAT were recorded in comparison with healthy subjects. PD patients involved in the present study had elevated CAT activity compared to healthy controls, and there were no differences between H&Y stages and the disease duration. Similar results were obtained in the research of Younes-Mhenni et al. [22], who have not observed the correlation between the duration of illness and CAT activity.

Several studies have shown contrasting results. Sudha et al. [27] observed no significant changes of erythrocyte antioxidants in PD patients while Abraham et al. [21] reported decreased AO enzymes activity in PD patients compared to controls. Considering that CAT is crucial in removing H_2O_2 at higher concentrations [35] (GPx is predominant at physiologically low levels of H_2O_2 [36]), elevation of CAT activity in the blood of PD patients confirms the general conclusion of this study that PD patients are exposed to chronic oxidative stress [37].

It is hypothesized that the adjustment of the AO system is based on shifts in AO activities rather than on the formation of new AO resources. Thus, for some aspects of the issue, it may be more useful to study whole groups of radical scavengers rather than focusing on individual molecule species [38]. PAB can be considered as a measure of an imbalance between oxidants (H_2O_2, tert-butylhydroperoxide, chloramine T and HClO) and antioxidants (vitamin C, trolox, GSH, uric acid, bilirubin, albumin, and ceruloplasmin) [39]. In our study, PAB shifted forward the OS indicating that PD patients had an elevated level of OS compared with healthy subjects, regardless of the H&Y stage.

The physiological roles played by the GSH include maintenance of thiol redox potential, clearing metabolic waste, and as a reservoir for amino acids [40]. Since GSH is involved in antioxidant defense and regulation of cellular metabolic functions ranging from gene expression, DNA, and protein synthesis to signal transduction, cell proliferation and apoptosis [41], its depletion might have a wide impact on many physiological and pathological processes. For instance, GSH deficiency has long been implicated in PD degeneration [42]. A recent report even suggests that whole blood GSH may have the utility as a biomarker in PD progression as it was statistically associated with PD status [43]. Accordingly, in our study, a blood concentration of GSH in PD patients was significantly decreased compared to healthy controls, and such tendency was more pronounced through H&Y stages. These findings are important as the changes in the level of GSH have consequences to numerous molecular processes as well as the progression of the disease. Furthermore, it should be emphasized that the exact cause of GSH reduction has not been fully clarified, however, it is known that the most common ways for reducing GSH involve its consumption by GPx, conjugation reaction with proteins[44] and 4-hydroxynonenal (4-HNE)[45] and translocation of GSH/GSSG across the plasma membrane[46]. In order to compensate for this decrease, the possible ways of therapeutic compensation of GSH are investigated. They include intranasal[47], intravenous, and liposomal[48] GSH augmentation, and some of them showed a promising effect in the treatment of PD disease[49].

Conclusion

Obtained results show that some of the examined AO parameters in blood of PD patients are possibly related to the PD stage. We observed a correlation of H&Y stage with PAB and AO parameters. The reduction of GSH level was associated with higher H&Y stage while PAB, SOD and CAT activity changed regardless of the H&Y score.

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Disclosure statement

The authors declare that there is no conflict of interests regarding the publication of this paper.

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