The Effects of Disease-Modifying Therapies on Oxidative Stress in Patients With Relapsing-Remitting Multiple Sclerosis

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Objective: Oxidative stress (OS) has a role in the pathogenesis and progression of multiple sclerosis. The effects of disease-modifying therapies (DMTs) on OS are unclear. We aimed to explore the association between DMTs and OS in patients with relapsing-remitting multiple sclerosis (RRMS).

Methods: The study conducted in 167 patients (102 received and 65 not received the DMTs). The DMTs included interferon beta-1a (n = 15), interferon beta-1b (n = 20), glatiramer acetate (n = 10), and sphingosine-1-phosphate receptor modulators (n = 57). Oxidative stress assessed by total antioxidant status (TAS) and total oxidant status (TOS) (determined by spectrophotometric method), oxidative index (OSI was calculated), and urinary 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG/creatinine was determined by high-performance liquid chromatography and tandem mass spectrometry). Patients were classified by Multiple Sclerosis Severity Score (MSSS) to mild/moderate (MSSS, <6.7) and severe (MSSS, >6.7).

Results: Disease-modifying therapies are associated with increased TAS, decreased TOS, OSI, and 8-oxodG/creatinine. Regardless of therapy, women had a less favorable redox status (lower TAS, higher TOS and OSI). Patients with MSSS>6.7 and without DMTs had higher OSI than patients who received DMTs. Women with MSSS>6.7 without DMTs had lower TAS than women with DMTs, whereas in the same stage of MS, men without DMTs had higher TOS than patients with DMTs. Women with MSSS<6.7 and with DMTs had lower 8-oxodG/creatinine compared with those without DMT therapy.

Conclusions: The antioxidant effects of DMTs were evidenced in this study. The gender-related effects of DMTs on the OS imply the personalized antioxidant pharmacotherapy, especially for the women. The OS biomarkers have a potential as the prognostic for the assessment of DMTs outcomes in patients with RRMS.

Key Words: multiple sclerosis, oxidative stress, interferon beta-1a, interferon beta-1b, sphingosine-1-phosphate receptor modulators

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Multiple sclerosis (MS) is a chronic, neurodegenerative, demyelinating, autoimmune disease of the central nervous system. Epidemiologic data has shown the rising prevalence of MS worldwide. The highest prevalence of MS in the world is in European region, with 133 patients per 100,000.[1] The prevalence of MS in Serbia is very close to the European average (136 per 100,000).[2] There are 3 forms of MS: relapsing-remitting MS (RRMS), primary progressive MS (PPMS), and secondary progressive MS (SPMS). The most common disease type is RRMS, characterized by periods of relapses, followed by remission periods.

The main hallmark of MS is chronic inflammation of central nervous systemt, which causes demyelination and subsequent neurodegeneration. In recent review article, Lassmann[3] summarized the current data about immunological and neurobiological mechanisms that may be involved in the onset and progression of MS. In this review, it was emphasized that the inflammation drives demyelination in all stages of the disease. Finally, the cascade pathways of oxidative injury, mitochondrial damage, and "virtual hypoxia" lead to demyelination and neurodegeneration, which are responsible for progression of MS. The question of what triggers oxidative and mitochondrial injury in MS patients is still open. The brain is sensitive to oxidative stress (OS) due to high production of reactive oxygen species (ROS) and reactive nitrogen species (RNS).[4] Oxidative stress is identified as a risk factor for onset and progression of MS and the main inducer of neurodegeneration and axonal damage.

Laboratory biomarkers for the assessment of the oxidative stress are total antioxidant status (TAS), total oxidant status (TOS), and oxidative index (OSI). The total antioxidant status of plasma (synonyms: antioxidant capacity) is assessed by inhibitory methods.[5,6] Total oxidant status involved measurement of oxidants present in the sample in oxidation reaction with various compounds.[7] The derived ratio of TOS/TAS, named as oxidative index (OSI), is a more reliable marker of the oxidant/antioxidant balance in the body.[8] The raising evidence is that biomarker of oxidative DNA damage, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) is potentially significant in MS pathology. This product of the DNA oxidation is biomarker of systemic effect of oxidative stress with the promutagenic potential.[9]

Although a cure for MS has not yet been discovered, the disease-modifying therapies (DMTs) for MS act to reduce the number and severity of MS relapses and slow down the rate of progression.[10] The treatment should be started soon after the diagnosis in order to reduce the occurrence of recurrence. Injectable DMTs used in the treatment of RRMS include interferon beta-1a, interferon beta-1b, and glatiramer acetate. The highly efficient group of DMTs is sphingosine-1-phosphate receptor modulators.

The aim of this study was to investigate the effect of DMTs therapies for MS on the oxidative stress biomarkers (TAS, TOS, OSI, 8-oxodG). The influence of the gender and the MS severity on the protective effect of DMTs on the OS development were also explored.

MATERIALS AND METHODS

Participants

The study included 167 RRMS patients, recruited from the Clinic of Neurology, Military Medical Academy, Serbia. Sample collection was performed between March 2018 and July 2019. The study has been approved by the local Ethics Committee (Ethics Committee of the Military Medical Academy, No. 4494-1) and was

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carried out according to the principles of the Helsinki Declaration. All participants gave written consent to participate in this study. Diagnosis was performed according to the diagnostic Mc Donald's Criteria.[11] The disease duration was measured in years, starting from the first MS sign and symptoms.

Disability of MS was evaluated by Expanded Disability Status Scale (EDSS).[12] The rates of disease progression were assessed by the Multiple Sclerosis Severity Score (MSSS), calculated by EDSS divided by the duration of disease.[13] Exclusion criteria were PPMS, diabetes mellitus, heart disease, and autoimmune diseases. Disease-modifying therapies received 102 patients and 65 patients were without therapy. Study was included 15 patients on the interferon beta-1a (Rebif), 20 patients on Interferon beta-1b (Betaferon), 10 patients on the Glatiramer acetate (Copaxone), and 57 patients on the sphingosine-1-phosphate receptor modulators (ponesimod, ozanimod, or fingolimod). The total duration of therapy (length of therapy) calculated by subtracting the date when patient started the MS therapy and the date when patient entered in this study. The average of total duration time of therapy was 5 years (range, 0.5–17.5 years).

Biochemical Analyses

Biomarkers of oxidative stress were analyzed in the serum and urine of MS patients. The serum was used to determine the OS biomarkers (TAS and TOS), and OSI parameter was calculated. The urine was used to determine 8-oxodG. Blood samples were collected after 12 hours of night fasting, using a Beckton & Dickinson Vacutainer Blood Collection Tubes. After spontaneous coagulation, blood samples were centrifuged at 850*g* (3000 rpm) for 15 minutes and serum aliquots were stored at −80°C until analysis. First-morning urine samples were centrifuged at 850*g* (3000 rpm) for 15 minutes and then were stored at −80°C until analysis. Before analysis, urine was left at 3°C to 8°C to thaw spontaneously. Then, they were homogenized on a vortex mixer and sonicated 5 minutes in an ultrasonic bath, centrifuged at 10,000*g* for 5 minutes.

The urinary 8-oxodG was determined using high-performance liquid chromatography and tandem mass spectrometry.[14] Measurement of 8-oxodG in urine was performed on Thermo Accela (Thermo Scientific, Waltham, MA), coupled to a triple quad Mass Spectrometer Thermo TSQ Quantum Access Max (Thermo Scientific) with a heated electrospray ionization (HESI) interface. All analyzed urine sample were mixed, sonicated for 5 minutes and then centrifuged for 10 minutes at 10,000*g*. Clear supernatants (20 μL) were then injected in a thermostatted high-performance liquid chromatography autosampler at 10°C. Results are expressed in relation to the concentration of creatinine in urine as nmol/mmol. The expression of 8-oxodG levels as an 8-oxodG/creatinine ratio was applicable when urinary creatinine excretion is constant. Analysis of the creatinine in urine was measured using a biochemical analyzer Advia 1200 (Siemens Healthcare Diagnostic, Tarrytown, NY) by the Jaffe kinetic method.

The serum TAS level was determined by modified Erel spectrophotometric method.[6] The reaction is based on the oxidation of a stable chromogen, 2,2′-azinobis-(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS), to the corresponding cation ($ABTS^{\cdot+}$) using hydrogen peroxide in an acidic environment, which creates an emerald color. The $ABTS^{+}$ is decolorized by antioxidants from the serum sample according to their concentrations, which is manifested by a decrease in color intensity at 660 nm. The intensity of discoloration is proportional to the concentration of total antioxidants in the sample. The method was applied on a biochemical analyzer Olympus AU400 (Beckman Coulter, Inc. USA). The reaction rate was calibrated with Trolox (6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid, the water-soluble analog of vitamin E), and results were expressed as mmol Trolox Equiv/L.

The TOS level was determined in serum samples using a modified Erel spectrophotometric method.[7] Oxidants from the sample oxidize ferrous iono-dianisidine complex to ferric ion in acidic medium. The oxidation reaction is facilitated by the presence of glycerol molecules in the reaction medium. Method was applied to the biochemical analyzer Olimpus AU400 (Beckman Coulter Inc. USA). The result was determined from the standard curve and expressed as μmol H_2O_2 Equiv/L.

In addition to the directly measured levels of TAS and TOS, the OSI was calculated using the formula: OSI (arbitrary unit)= [TOS (mmol H_2O_2 Eq/L)/TAS (mmol Trolox Eq/L)]×100.[8]

STATISTICAL ANALYSIS

The Kolmogorov-Smirnov test was used to examine the normality of the data distribution. If the data showed a normal distribution, they were displayed as the mean ± standard deviation, in that case, parametric statistical tests of comparison of mean values between two groups (the independent-samples *t* test). A probability value less than 0.05 was considered statistically significant. The IBM SPSS Statistics for Windows, Version 20.0 was used for the statistical analysis.

RESULTS

Demographic Characteristics and Parameters of Oxidative Stress in MS Patients

The demographic characteristics and parameters of oxidative stress in MS patients are shown in Table 1. Patients who did not receive the DMTs had significantly higher MSSS score ($P < 0.001$), lower level of TAS ($P = 0.033$), and higher levels of urinary 8-oxodG/creatinine ($P = 0.003$). The gender-related differences of OS parameters were obtained regardless of DMTs. Higher levels of TAS were obtained in men than in women (patients without DMTs, $P = 0.006$; patients with DMTs, $P < 0.001$). However, the higher levels of TOS and OSI were obtained in women than in men (TOS: patients without DMTs, $P = 0.017$; patients with DMTs, $P = 0.003$; OSI: patients without DMTs, $P = 0.017$; patients with DMTs, $P < 0.001$). The gender-related effects of DMTs on the OS parameters were analyzed in four groups of patients (men without DMTs, n = 21; men with DMTs, n = 53; women without DMTs, n = 44; women with DMTs, n = 49), and results were shown in Table 1. Urinary 8-oxodG/creatinine levels were significantly higher in women without DMTs than in women who received DMTs ($P = 0.014$).

Association Between MS Severity, Disease-Modifying Therapies and Oxidative Stress

The investigation of the gender-related effects of DMTs on the OS parameters according to the disease severity tested using the independent-samples *t* test, and results presented in Figure 1. Patients were divided into groups: patients with mild and moderate MS (MSSS<6.7) and without DMTs (men, n = 11; women, n = 21); patients with mild and moderate MS (MSSS<6.7) who received DMTs (men, n = 44; women, n = 38); patients with severe MS (MSSS>6.7) without DMTs (men, n = 3; women, n = 7); and patients with severe MS (MSSS>6.7) with DMTs (men, n = 8; women, n = 8). Patients with severe MS (MSSS>6.7) and without DMTs had higher values of OSI than the groups with DMTs, regardless of gender (men, $P = 0.031$; women, $P = 0.057$) (Fig. 1C). In group of women, patients without DMTs and MSSS>6.7 had lower TAS than patients with DMTs ($P = 0.041$) (Fig. 1A), whereas

TABLE 1. Demographic, Clinical Characteristics and Oxidative Stress Parameters in Patients With RRMS, According to Gender and Received DMTs

	Patients Without DMTs			Patients With DMTs		
Parameters	**All (N = 65)**	**Males (n = 21)**	**Females (n = 44)**	**All (N = 102)**	**Males (n = 53)**	**Females (n = 49)**
Age, y	39.7 ± 11.1	38.9 ± 8.4	40.1 ± 12.0	41.9 ± 9.2	42.6 ± 8.5	41.1 ± 10.0
Length of therapy, y	—	—	—	5.0 ± 3.8	5.7 ± 3.8	4.5 ± 3.7
Males, n (%)	21 (32) †	—	—	55 (54)	—	—
EDSS	2.8 ± 1.6	2.7 ± 1.9	2.9 ± 1.4	2.5 ± 1.2	2.5 ± 1.2	2.6 ± 1.2
MSSS	5.1 ± 2.3 †	4.7 ± 2.6 †	5.3 ± 2.2 †	3.7 ± 2.2	3.4 ± 2.2	3.9 ± 2.1
TAS (mmol Trolox Equiv/L)	1.96 ± 0.18 †	2.05 ± 0.19 *	1.92 ± 0.16	2.03 ± 0.22	2.11 ± 0.21 *	1.94 ± 0.19
TOS (μmol H_2O_2 Equiv/L)	9.10 ± 1.88	8.31 ± 1.79 *	9.48 ± 1.81	8.97 ± 1.70	8.64 ± 1.62 *	9.32 ± 1.73
OSI (arbitrary units)	0.47 ± 0.11	0.41 ± 0.11 *	0.50 ± 0.10	0.45 ± 0.10	0.41 ± 0.86 *	0.48 ± 0.10
8-oxodG/creatinine (nmol/mmol)	1.84 ± 8.97 †	1.67 ± 0.59	1.92 ± 1.10 †	1.38 ± 0.75	1.38 ± 0.74	1.38 ± 0.77

Continuous variables are shown as mean ± standard deviation.

*Differences between men and women.

†Differences between patients who received and did not receive DMTs therapy.

in the group of men, patients without DMTs and MSSS>6.7 had higher TOS than patients who received DMTs (P = 0.033) (Fig. 1B). Women with MSSS<6.7, who received DMTs had lower 8-oxodG/creatinine (P = 0.041) compared with those without DMTs therapy.

DISCUSSION

Oxidative stress was identified as the one of the etiological factors in MS.[4] In a very recently review article was summarized the contribution of inflammation and oxidative stress to the pathology of MS, and discussed about the consequences to the development of therapy for MS.[15] Literary data about the effects of DMTs on oxidative stress are very scarce. Since that, the antioxidative supplements are including in the MS management, the development of new antioxidative therapeutics in the treatment of MS should be consider. To the best of our knowledge, this is the first study that investigated the effect of DMTs therapy on oxidative stress in RRMS patients in population of Serbia. We found that the patients without DMTs therapy had higher Multiple Sclerosis Severity

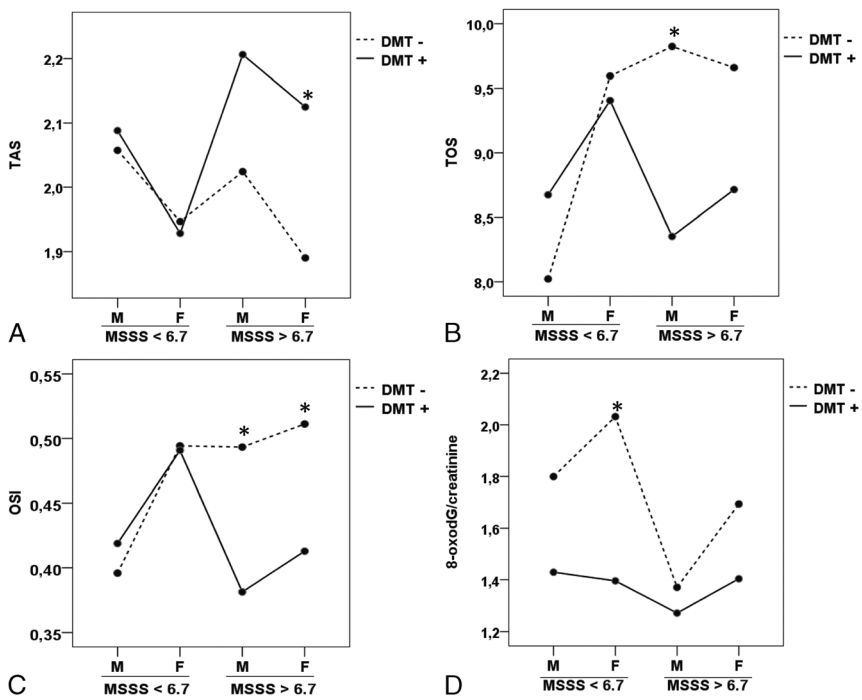

FIGURE 1. Gender-related (M, males; F, females) estimated marginal means of OS parameters in groups: patients with mild or moderate MS without DMTs (MSSS<6.7, DMTs –); patients with mild or moderate MS with DMTs (MSSS<6.7, DMTs +); patients with severe MS without DMTs (MSSS>6.7, DMTs –); patients with severe MS with DMTs (MSSS>6.7, DMTs +); (A) TAS (mmol Trolox Equiv/L); (B) TOS (μmol H_2O_2 Equiv/L); (C) OSI (arbitrary units); (D) 8-oxodG/creatinine (nmol/mmol); *Differences between patients who received and did not receive DMTs (P < 0.05).

Score (MSSS), higher level of 8-oxodG/creatinine, and lower level of TAS, compared with the group of patients who received DMTs. This may imply that antioxidant effects of DMTs contribute to the less disability and slower progression of the disease. This effect of DMTs is important because research have shown increased prooxidative and decreased antioxidative biomarkers in active plaque, white matter and gray matter from patients with MS.[16,17] Specifically, a significant increased level of 8-oxodG was found within chronic active plaques when compared with the normal white matter regions in the same cerebellum from MS patients who suffered from severe cerebellar symptoms during the course of the disease.[18] Moreover, it was demonstrated that the oxidative injury of oligodendrocytes and neurons are associated with active demyelination and axonal or neuronal injury in MS.[19] In that study, immunoreactivity for 8-hydroxy-D-guanosine (oxidized DNA lesion) was mainly found in areas with profound microglia activation at the lesion edge ("initial" lesion area). In addition to the immunocytochemistry detection of oxidized DNA products in brain tissue, in the study of Tasset et al[20] was founded the higher level of peripheral 8-oxodG in plasma samples of RRMS patients than in control group. In our study, the finding of increased urinary 8-oxodG/creatinine in untreated RRMS patients was in agreement with these studies and probably indicates serious systemic effects of oxidative stress in patients with RRMS. Furthermore, we noticed that women regardless of therapy had lower level of TAS, higher level of TOS and OSI than men. Hence, we suppose that the women with RRMS have a higher risk for developing oxidative stress than men, which manifested by decreased antioxidative and increased prooxidative biomarkers. Our data corresponded with the findings of lower antioxidative biomarkers (CoQ10, antioxidant power), and higher reactive oxygen species in female patients than in male patients with MS.[21] Also, low serum urate levels (major endogenous antioxidant) were obtained in early stage of MS, predominantly in women.[22] Our finding that women had lower TAS regardless of therapy is in agreement with these studies. Despite the elevated oxidative stress in the extracellular space (assessed by the TAS, TOS, and OSI in serum), we found that DMTs in women are associated with decreased level of urinary 8-oxodG/creatinine, which is predominantly intracellular oxidative stress biomarker. In general, population of Serbia, women had higher levels of urinary 8-oxodG/creatinine than men, which supports the finding that women are more susceptible to oxidative stress.[14] Considering to the greater sensitivity of women to oxidative stress, it can be assumed that the early initiation of DMTs would be especially important for women. Epidemiological studies have confirmed that the incidence of MS is at least twice as high in women in the most of the world's populations.[1] This gender dimorphism motivates many investigators to clarify how genetics, environment and other factors increase a woman's chances of developing MS, with the aim to reveal a new way to treat or even prevents the MS. We speculate that the increased sensitivity of women to oxidative stress may be responsible for the gender dimorphism in MS prevalence, considering the significant role of oxidative stress in the pathogenesis of this disease. In the detailed systematic review, which presented 14 studies about gender difference in the efficacy of DMTs, therapy was not found the clear gender-related differences in response to DMTs.[23] Our finding that the DMTs are associated with the decreased level of 8-oxodG/creatinine in women can contribute to the clarification of this issue. On the other hand, the elevated urinary 8-oxodG in women without therapy indicates systemic and cumulative effect of oxidative stress, which possibly makes them susceptible to a worse course of the disease. Therewithal, in the management of MS, the combination of DMTs and antioxidants (as dietary prophylaxis or pharmacological antioxidative therapies) could additionally prevent the spreading of brain tissue damage and delay of the MS progression. In a recent review article, various antioxidant compounds were presented and their potential utility as complementary therapy in MS.[24] The conclusion of that review article pointed out the necessity of further research to understand the potential protective effects of antioxidants against neurodegeneration in MS.

In addition, we noticed the elevated susceptibility of women on oxidative stress, examining the gender-related effect of DMTs on the oxidative stress depending on the severity of the disease. The DMTs showed protective effect against the OS, manifested as the lower OSI in group of patients with severe MS (MSSS>6.7) in both genders. The DMTs in women were increased the level of TAS, but DMTs were decreased the levels of TOS in men. In addition, DMTs prevented the increase of 8-oxodG/creatinine in women with mild and moderate MS (MSSS<6.7). The protective role of DMTs in the female population may be important, even in the early phase of disease. That implies usefulness of OS parameters (TAS, TOS, OSI, and 8-oxodG/creatinine) in evaluation of oxidative stress in prevention of severe stage of MS, during the early application of DMTs. Our data could be supported by the histopathological findings that oxidative stress is crucial in the early stages of MS.[25] Taking into consideration the above, we can hypothesize that the DMTs have protective effect on oxidative stress developing, and probable slower course of MS progression, especially in women.

CONCLUSIONS

The current study demonstrates the antioxidant effects of DMTs in patients with RRMS. Regardless that the main limitation of this study was a small number of MS patients, the obtained gender-related effects of DMTs on the oxidative stress may be clinically significant. Application of DMTs therapy in early phase of MS probably prevent the oxidative stress development and therefore progression of the disease, especially in women, but this finding requires further studies. Results of this study may encourage further investigation of personalized antioxidant pharmacotherapy as the support especially for the female population on the DMTs. Improvement of laboratory evaluation of DMTs therapy outcomes should include the traditional OS biomarkers (TAS, TOS, and OSI) and biomarker of DNA oxidation (8-oxodG/creatinine).

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