Nitrosative stress parameters and the level of oxidized DNA bases in patients with multiple sclerosis

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Abstract

Multiple sclerosis (MS) is a neurodegenerative disease with various factors affecting its etiology. Overproduction of nitric oxide and subsequent lesions of biopolymers are some of the possible causes of the disease. This study aimed to measure the most relevant nitrosative and oxidative stress biomarkers and the level of modified DNA bases in patients with MS. Each parameter was assayed in 25 patients with MS and 25 healthy controls. This study involved detecting blood plasma and serum nitric oxide metabolites by chemiluminescence detector Sievers NOA-280i, malondialdehyde (MDA) measurements with thiobarbituric acid reactive substance (TBARS) assay, detection of oxidized purines and pyrimidines with the enzyme-modified comet assay. Statistical analysis of the results was performed by one-way analysis of variance (ANOVA) and unpaired t test for the comparison of less than three data sets. DNA single-strand breaks, levels of modified purines and pyrimidines, as well as nitrite and nitrate levels in plasma and serum samples, were significantly higher in patients with MS than in healthy controls. On the contrary, MDA levels appeared to be lower in patients with MS.

1. Introduction

Multiple sclerosis is a neurodegenerative disease with chronic and inflammatory characteristics. MS targets axons in the CNS, causing their demyelination, thus disrupting signaling pathways (Stadelmann et al., 2011; Trapp and Nave, 2008). A total of 2.8 million people are estimated to live with MS worldwide (Walton et al., 2020). While symptoms can occur at any age, the most common onset age is usually 20 to 40 years (Magyari and Sorensen, 2019).

The exact cause of the demyelination is still unknown. This process is supposed to develop as the sequence of various factors such as environmental, genetic, metabolic, viral, or oxidative stress-induced. Together or separately, these factors result in an autoimmune disorder with the consequent immune attack on the CNS (Fiorini et al., 2013; Gonsette, 2008; Miller et al., 2012).

Multiple sclerosis is classified into three main subtypes based on the clinical course of the disease. Relapsing-remitting Multiple Sclerosis (RRMS) is the most common type of ailment. It is characterized by relapses of symptoms following the improvement in the patient's general condition or total disappearance of symptoms. Secondary progressive MS (SPMS) develops as the following stage of the RRMS, and it is characterized by a lack of remissions or gradual deterioration of the patient. Primary progressive MS (PPMS) appears in the form of gradual worsening of symptoms with occasional stability in overall condition. (Ghasemi et al., 2017; Goldenberg, 2012; Kalincik, 2015; Thompson et al., 2018; Weiner, 2008). Alternatively MS phenotypes can be classified according the Expanded Disability Status Scale (EDSS) (Lublin et al., 2014).

Various studies have already confirmed the theory about oxidative stress being a crucial factor in the demyelination process in MS. (Fiorini et al., 2013; Gonsette, 2008; Miller et al., 2012). Oxidative stress level can be assessed determining the molecules changed under the influence of reactive oxygen species

(ROS) and reactive nitrogen species (RNS). Furthermore, under the effect of oxidative stress, deoxyguanosine is rapidly converted to 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) (Hinokio et al., 1999).

Overproduction of nitric oxide by inducible NO synthase (iNOS) in oligodendrocytes with subsequent lesions of mitochondria is considered crucial in the pathogenesis of MS (Lan et al., 2018). Microglia-mediated neuroinflammation is deemed to be a priming event in the onset and progression of MS. Microglia activation is associated with the release of proinflammatory cytokines and increased production of superoxide and nitric oxide. The two radicals then react to form the neurotoxic peroxynitrite (Kumar et al., 2014). Overproduction of NO in the brain can influence the level of NO metabolites – nitrites and nitrates in the blood (Adamczyk et al., 2017).

ROS-mediated process of lipid peroxidation results in the degradation of polyunsaturated lipids. Reaching biological membranes, this chain of reactions can be induced by various toxic products, including endoperoxides and aldehydes (Rosenblum et al., 1989). MDA is one such secondary product and is commonly used as a biomarker for cell membrane damage evaluation (Esterbauer et al., 1991).

The present study aimed to evaluate the oxidative stress damage in peripheral blood mononuclear cells, measurements of NO metabolite, and lipid peroxidation products in human plasma and serum.

2. Materials And Methods

2.1. Patients

Permission of the Central Medical Ethics Committee of the Republic of Latvia No 1/17-10-10 was received to perform this study. Informed consent was obtained from every participant of the study. The study group consisted of 25 unrelated patients with MS randomly chosen among the MS patients attending the Latvian Maritime Medicine Center (detailed information is summarized in Table 1) and the control group of 25 healthy subjects: 4 males and 21 females of 20 to 43 years (average age of the group 34.2 ± 1.7 years). Healthy subjects were volunteers and were defined as those who did not have a history of any chronic and autoimmune diseases. Collection of the MS and control group samples was performed during the same season, samples of both groups were analysed in parallel. Most of the patients were undergoing disease modifying therapies (DMT). Drugs were chosen according to individual preference, availability on the market and effectiveness. Samples of the same patients were used for every method.

2.2. Collection and processing of blood samples

Blood was obtained by vein puncture and collected in plastic capillaries with EDTA (BD Vacutainer K2E EDTA 10.8 mg, BD-Plymouth, UK). Peripheral blood mononuclear cells (PBMNC) were isolated using Histopaque-1077 (Sigma-Aldrich, Germany) according to the protocol provided by the manufacturer.

Plasma preparation. Whole blood was collected into EDTA treated tubes and centrifuged for 10 minutes at 1500 x g in the pre-cooled centrifuge. The supernatant was removed, split into 0.5 mL aliquots, and stored at -20 °C.

Serum preparation. Whole blood was collected into EDTA-free tubes and allowed to clot at room temperature. The clot was removed by centrifugation for 10 minutes at 1500 x g in the pre-cooled centrifuge. The supernatant was immediately split into 0.5 mL aliquots and stored at -20 °C

2.3. Modified single-cell gel electrophoresis (modified comet assay)

The DNA single-strand damage and oxidized bases were detected in isolated peripheral blood mononuclear cells utilizing single-cell gel electrophoresis in the presence of two DNA repair enzymes: Fpg (Sigma-Aldrich, Germany) and EndoIII (Sigma-Aldrich, Germany). This modification of the comet assay enables detection of the oxidized bases (A. R. Collins et al., 1993; Collins et al., 1996). Control and patient samples were analysed under the same conditions. Each of them was analysed in duplicates – two samples per slide. The general procedure for alkaline comet assay was previously described (Borisovs et al., 2019) and performed with a few modifications. 50 μL of isolated PBMNC were mixed with 100 μL of 1% low-melting agarose preheated to 37 °C (Sigma-Aldrich, Germany). 50 μL of the mixture were placed on an agarose-precoated (0.5% type III, Sigma-Aldrich, Germany) microscope slide (two samples per one slide) and covered with 24 x 24 mm cover glass (Corning, USA). The slides were kept on the ice up to the solidification of agarose. 10 mM $KBrO_3$ solution was prepared (0.1 N $KBrO_3$ stock, Merck, Germany), 50 μL of this solution were applied on the prepared sample slides, covered with cover glass, and incubated for 5 minutes at room temperature. Slides were placed into the lysis solution (2.5 M NaCl, 10 mM Na2EDTA, 10 mM Tris, pH 10 [AppliChem, Germany], 1% Triton-X 100, 5% DMSO [Sigma-Aldrich, Germany]) and left overnight at + 4 °C. On the next day and right before use, fresh enzyme reaction (ER) buffer was prepared: 40 mL HEPES (Sigma-Aldrich, Germany), 0.1 M KCl (Sigma-Aldrich), 0.5 mM EDTA (Sigma-Aldrich, Germany), 0.2 mg/mL BSA (Sigma-Aldrich, Germany), pH 8. Fpg protein from E. coli and EndoIII protein from E. coli 1:3000 dilutions in ER buffer were prepared. 50 μL of each enzyme solution were applied to the samples. Samples were then incubated at 37 °C: 30 minutes for Fpg and 45 minutes for EndoIII. After the incubation, the standard alkaline comet assay protocol was followed. Cells were visually graded into 5 classes (A0 – A4) (Collins et al., 1993; Collins et al., 1994) from class 0 (undamaged, no discernible tail) to class 4 (almost all DNA in tail, insignificant head). DNA damage index (D) in arbitrary units was calculated as follows: D = A1 + 2 × A2 + 3 × A3 + 4 × A4.

2.4. Nitrite and nitrate measurements

Chemiluminescence detector Sievers NOA-280i was used for the following analyses.

Measurements of nitrites. This and the following procedure were performed according to the manual provided by the manufacturer. 5 mL of the reducing agent were prepared by dissolving 50 mg NaI (Sigma-Aldrich, Germany) in 0.5 μL deionized water and mixing with 4.5 mL glacial acetic acid (Sigma-Aldrich, Germany). 10 mL of 100 mM NO_2^- standard stock solution were prepared by dissolving ~ 69 mg $NaNO_2$

(Sigma-Aldrich, Germany) in 10 mL deionized water. 10 nM NO_2^-, 100 nM NO_2^-, 1 µM NO_2^- and 10 µM NO_2^- standard solutions were prepared by diluting the 100 nM standard stock solution. 50 µL of each standard solution were injected in triplicates, and a calibration curve was created before the experiment. 50 µL of each plasma or serum sample were injected into the reducing agent.

Measurements of nitrates. 100 mL of the reducing agent were prepared by dissolving 0.8 g VCl_3 (Sigma-Aldrich, Germany) in 1 M HCl (Sigma-Aldrich, Germany) and filtered through the filter paper. The gas bubbler was filled with 15 mL of 1 M aqueous NaOH (Sigma-Aldrich, Germany) solution to prevent HCl vapours from entering the NOA. The water bath was connected to the purge vessel, and the temperature was set to 95 °C. 10 mL of 100 mM NO_3^- standard stock solution were prepared by dissolving ~ 85 mg $NaNO_3$ (Sigma-Aldrich, Germany) in 10 mL deionized water. 10 nM NO_3^-, 100 nM NO_3^-, 1 µM NO_3^- and 10 µM NO_3^- standard solutions were prepared by diluting the 100 nM standard stock solution. 50 µL of each standard solution were injected in triplicates, and a calibration curve was created before the experiment. 50 µL of each plasma or serum sample were injected into the reducing agent and NO_3^-.

Concentrations of NO_2^- and NO_3^- were calculated using the provided "NOA Liquid" software based on each calibration curve.

2.5. TBARS assay

Measurements were performed by Lambda 25 UV-VIS spectrometer (PerkinElmer, UK) in 1 cm thick single-use cuvettes (Sarstedt, Germany) 4.0 mM TBA standard solution was prepared by dissolving 57.66 mg TBA (Sigma-Aldrich, Germany) in 100 mL 99.5% glacial acetic acid (Sigma-Aldrich, Germany). 1 mM MDA standard stock solution was prepared by dissolving 31.35 mg malondialdehyde tetrabutylammonium salt (Sigma-Aldrich, Germany) in 100 mL 99.5% glacial acetic acid. 0.1, 0.2, 0.4, 0.6, and 0.8 mM standard solutions were prepared by diluting the 4 mM MDA standard stock solution. 500 µL of 4.0 mM standard TBA solution were mixed with the corresponding MDA standard solution, and the mixture was heated for 60 minutes at 95 °C in the water bath. The absorption of each sample was then measured at 532 nm, and the calibration curve was constructed.

500 µL of plasma or serum were mixed with 500 µL 4.0 mM TBA standard solution. The mixture was then heated for 60 minutes at 95 °C in the water bath, and the absorption of each sample was measured at 532 nm. MDA concentration in plasma or serum samples was calculated based on the calibration curve.

2.6. Statistical analysis

All the data of DNA damage levels assayed by the enzymatic comet assay were measured in arbitrary units (AU) represented as mean ± standard error of the mean (SEM). Ordinary non-parametric one-way analysis of variance (ANOVA) was performed using the commercially available GraphPad Prism, and the data were considered statistically significant at $p < 0.05$.

3. Results

3.1. Demographic data

Samples from the same 25 patients with MS (study group is described in Table 1), and 25 healthy subjects were used in each experiment. RRMS was the predominant type of MS in the study group. Table 2shows the disease-modifying therapy (DMT) synopsis based on administration of the active substance of each drug. Taking into account the total number of the registered MS patients in Latvia (2035), 1.2% of all patients were enrolled in the study.

Table 1

Demographic and clinical characteristics of patients with MS in the study group

	RRMS	SPMS
Patients	21	4
Age, years	43.2 ± 12.5	46.3 ± 9.7
Male/female	7/14	2/2
Disease duration, years, (IQR in years)	7.7 (4.3−10.8)	8.5 (6.5−10.5)
Average EDSS score	3.7 ± 1.0	3.6 ± 1.0
Spinal cord lesions on MRI (yes/no)	21/0	4/0
Brain lesions on MRI (yes/no)	21/0	4/0

EDSS – Expanded Disability Status Scale, IQR – interquartile range, RRMS – relapse–remitting multiple sclerosis, SPMS – secondary progressive multiple sclerosis, MRI – magnetic resonance imaging
All data are presented in mean values unless otherwise stated·

Table 2

Demographic and clinical characteristics of the study group, according to different disease-modifying therapies

Parameter	DMF	IFN-β1a	mAb	PEGIFN-β1a	TF	IFN-β1b	GA	No
N	2	8	2	2	5	2	2	2
Age, years	37.5 ± 13.5	42.3 ± 9.5	33.0 ± 1.0	39.0 ± 10.0	39.4 ± 7.5	48.0 ± 4.0	59.0 ± 5.0	63.0 ± 9.0
Start of DMT, years	27.5 ± 8.5	31.0 ± 9.6	23.5 ± 1.5	30.0 ± 7.0	26.8 ± 5.9	37.5 ± 3.5	45.5 ± 5.5	-
% of females	0	75	100	100	60	100	50	0
Duration of DMT, years	11.0 ± 5.0	13.4 ± 1.8	9.5 ± 0.5	9.0 ± 3.0	12.8 ± 2.3	10.5 ± 0.5	13.5 ± 0.5	-
EDSS, score	2.25 ± 0.35	4.25 ± 1.38	2.50 ± 0.00	2.75 ± 0.25	3.20 ± 1.29	4.25 ± 0.75	3.00 ± 0.00	5.00 ± 2.00

DMF – Dimethyl fumarate; IFN–β1a – interferon beta (IFN–β1a is produced by mammalian cells; mAb – monoclonal antibody; PEGIFN–β1a has a polyethylene glycol (PEG) attached) TF – Teriflunomide; IFN–β1b is produced in modified E. coli; GA – glatiramer acetate, No – no treatment; DMT – disease–modifying therapy·

3.2. Modified comet assay

Comet assay results (Fig 1 – 3 and 4) show an increased level of DNA single-strand breaks in patients with MS compared with the healthy subjects. This observation supplements previous alkaline comet assay results (Borisovs et al., 2019). To evaluate the number of oxidized bases, DNA damage data was subtracted from the enzyme induced damage data. Apparently, the level of oxidized bases is similar in the MS (Fig. 1–5 and 6) and control groups (Fig. 1–3 and 4). Individual deviations between patient were highly variable, statistically significant differences were not reached ($p = 0.919$).

3.3. TBARS assay in plasma and serum

MDA is a widely used biomarker for various disorders, including MS. Results shown in Fig. 2 indicate a decrease in MDA levels in plasma and serum of patients with MS compared to healthy subjects. Plasma and serum control samples have 5.33 ± 0.19 µM MDA (Fig. 3; Control group – P) and 6.07 ± 0.42 µM MDA (Fig. 2; Control group – S) accordingly. As for the plasma and serum from patients with MS – 3.78 ± 0.32 µM MDA (Fig. 2; patients with MS – P) and 4.95 ± 0.23 µM MDA (Fig. 2; Patients with MS – S) accordingly.

3.4. NO metabolites in plasma and serum

To our knowledge, there is currently no evidence proving whether it is more accurate to determine metabolite concentrations in blood plasma or serum. In this study, nitrite and nitrate concentrations were

determined both in plasma and serum of patients with MS. According to results shown in Fig. 3, nitrite concentration in plasma and serum of healthy subjects are on the same level − 0.64 ± 0.02 µM (Fig. 3; Control group − P) in plasma and 0.65 ± 0.04 µM (Fig. 3; Control group − S) in serum. On the other hand, overall levels of nitrites in plasma and serum of patients with MS are significantly higher than in healthy subjects. However, nitrite concentration in plasma appeared to be higher than in serum − 1.13 ± 0.05 µM (Fig. 3 Patients with MS - P) in plasma and 0.79 ± 0.06 µM (Fig. 3; Patients with MS - S) in serum.

Nitrate concentrations (Fig. 4) follow a similar trend to nitrite concentrations. Namely, both plasma and serum of healthy subjects have the same levels of nitrates − 24.98 ± 1.20 µM (Fig. 4; Control group - P) in plasma and 25.05 ± 3.44 µM (Fig. 4; Control group - S) in serum. Samples taken from patients with MS indicate higher nitrate amounts both in plasma and serum − 33.67 ± 2.97 µM (Fig. 4; Patients with MS - P) in plasma and 36.30 ± 3.81 µM (Fig. 4; Patients with MS - S) in serum.

4. Discussion

According to different studies subjects with diagnosed Alzheimer's (AD) and Parkinson's (PD) diseases have higher levels of single-strand DNA breaks (Milic et al., 2015). Another set of studies described by Kuchařová et al., (2019) support the same idea and indicate that patients with AD and PD also have higher levels of oxidized DNA bases. Interestingly, Souliotis et al., 2019 describe the correlation between the increase of DNA damage and polymorphisms at DNA repair enzymes' genes of patients with Systematic Sclerosis. DNA damage levels are quite variable for each patient, and so are levels of oxidized bases. However, even though current data is not statistically significant, control group had about the same levels of oxidized bases as the study group. Considering overall DNA damage is higher within patients with MS, bigger sample pool is required to evaluate the potency of oxidized bases as a biomarker for MS.

Adamczyk et al., (2017) conducted research evaluating MDA levels in serum of newly diagnosed (untreated) patients with MS and depending on three different drug therapies. Their results indicate that newly diagnosed patients with RRMS have the highest amount of MDA in serum. Drug-treated patients have lower amounts of MDA in their serum, but values are still higher than control samples. Lower MDA levels contradict previous research performed by Saif Eldeen et al., 2019, which states that patients with MS have increased MDA levels in serum. On the other hand, research by Noroozi et al., (2017) has shown a significant decrease in patients' serum MDA after a period of IFN-β 1a therapy.

The amount of data regarding NO metabolites, namely, nitrites and nitrates, is currently scarcy. One of the first studies on the given topic shows an increase in nitrite concentration in the human serum of patients with demyelination disorders (Giovannoni et al., 1997).

Examples include acute disseminated *encephalomyelitis* and *neuromyelitis optica*. The nitrite and nitrate levels in serum of *neuromyelitis optica* patients were previously reported to match the NO_x levels in

patients with MS (Haghikia et al., 2015). Acute disseminated *encephalomyelitis* is characterized by a profound increase in NOx serum levels than those found in patients with MS (Fominykh et al., 2016).

The primary assumption at this point is that an increase of NO metabolites in blood plasma and serum of the patients might be due to the overproduction of NO by iNOS in oligodendrocytes (Lan et al., 2018).

Overall, the increased levels of NO_x in the serum of patients with MS reported in the present study are consistent with previously reported NO_x levels for at least several other demyelinating diseases.

Levels of NO metabolites were significantly higher in MS patients. This leads to an assumption that the overproduction of nitric oxide synthases might be the case for MS. No objective evidence to assume the effect of DMTs on the overproduction of NOS.

In the case of MDA, previously described studies indicate that MDA levels should generally be higher within patients of MS, so in this case, DMTs most-likely influence the overall MDA levels. Further investigation with larger study group undergoing DMTs is required.

5. Conclusions

- Patients with MS have higher overall DNA damage levels, but oxidized bases appear to be on the same level as in the control group.
- Compared to healthy subjects, patients with MS have decreased MDA levels both in plasma and serum samples.
- Nitrite concentrations in plasma and serum of patients with MS are significantly higher compared to healthy subjects.
- Nitrate concentrations in plasma and serum of patients with MS are on similar levels and significantly higher than in healthy subjects.

Declarations

Conflicts of interest

No potential conflicts of interest were declared regarding the study, authorship, and publication of this article.

Ethics approval

Permission of the Central Medical Ethics Committee of the Republic of Latvia No 1/17-10-10 was received to perform this study. Informed consent was obtained from every participant of the study.

Data availability

All data generated or analyzed during the study are summarized into figures and included in the manuscript.

Code availability

Not applicable.

Authors' contribution

V. Borisovs designed the study as a part of his Ph.D. thesis. V. Borisovs and J. Bodrenko performed experiments. Samples were received from patients with MS under the supervision of J. Kalnina. V. Borisovs wrote the manuscript, and N. Sjakste revised the final version. All authors have read and approved the final manuscript.

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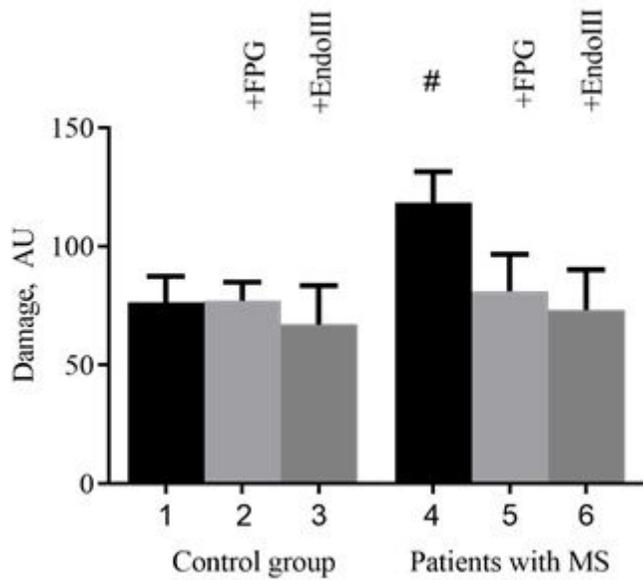
Figures

Figure 1

Modified comet assay data showing DNA damage levels and levels of oxidized bases (arbitrary units) in PBMNC of healthy controls and patients with MS. 1 – Total DNA damage of healthy subjects; 2 and 3 – levels of oxidized bases in healthy subjects; 4 – total DNA damage of patients with MS; 5 and 6 – levels of oxidized bases in patients with MS. Data are presented as mean ± SEM (arbitrary units). # $p < 0.05$ vs. the control group; levels of oxidized bases were not statistically significant ($p = 0.9190$)
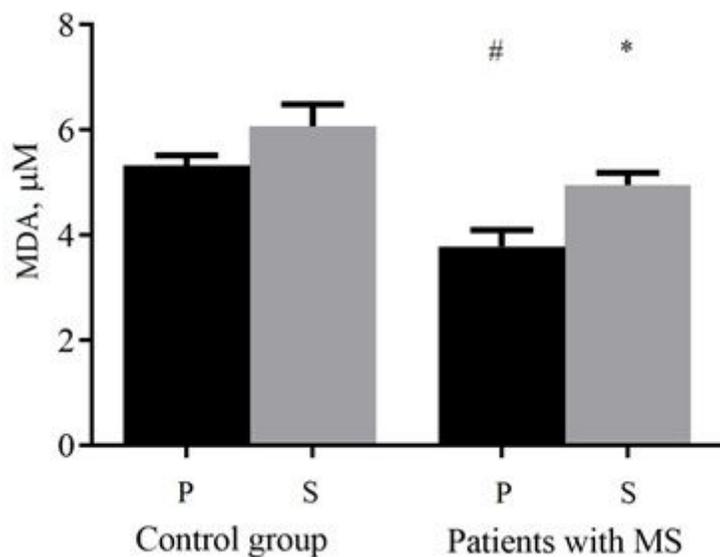

Figure 2

Concentrations of MDA, according to TBARS assay determined in human blood plasma and serum of patients with MS and healthy subjects. P and S – plasma and serum samples. Data are presented as mean ± SEM (μM). # and * $p < 0.05$ vs. the control group
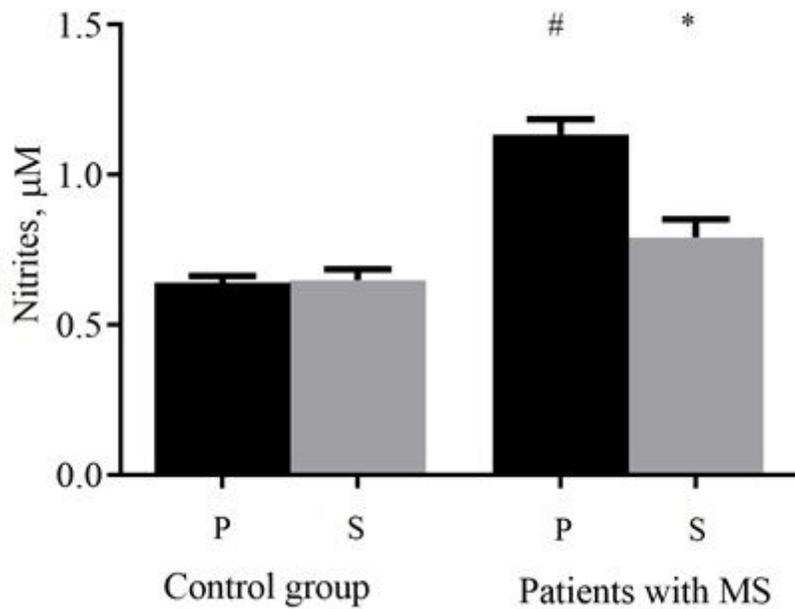
Figure 3

Concentrations of nitrites determined in human blood plasma and serum of patients with MS and healthy subjects. P and S – plasma and serum samples correspondingly. Data are presented as mean ± SEM (µM). # and * $p < 0.05$ vs. the control group
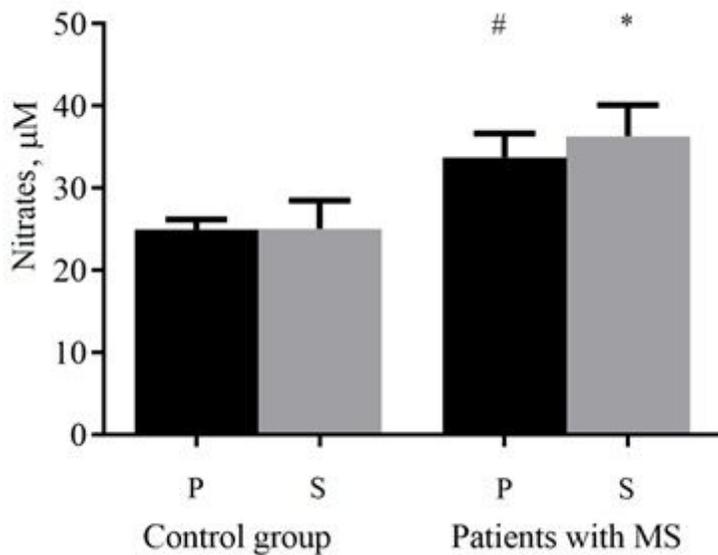

Figure 4

Concentrations of nitrates determined in human blood plasma and serum of patients with MS and healthy subjects. P and S – plasma and serum samples. Data are presented as mean ± SEM (µM). # and * $p < 0.05$ vs. the control group