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Serum NADPH oxidase concentrations and the associations with iron metabolism in relapsing remitting multiple sclerosis

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ABSTRACT

Background: Overproduction of reactive oxygen species (ROS) and impaired iron metabolism are considered to be possible factors in the pathogenesis of Multiple sclerosis (MS). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases are the primary sources of regulated ROS production. The NADPH oxidase (NOX) family consists of seven catalytic homologues, NOX1–5 and two dual oxidases. NOX1 and NOX5 are associated with endothelial dysfunction and inflammation but NOX4 has a protective effect on vascular function. The aims of this study were to investigate the status of NOX1, NOX4 and NOX5 and its relationship with serum iron metabolism biomarkers in relapsing-remitting MS patients.

Methods: The study included 53 RRMS patients and 45 control subjects. Serum NOX1,4,5, ferritin, iron, unbound-iron binding capacity, C-reactive protein (CRP), white blood count (WBC) and erythrocyte sedimentation rate (ESR) levels were measured in all the study subjects.

Results: Higher serum NOX5 ($p < 0.0001$), CRP ($p = 0.014$), ferritin ($p = 0.040$) and lower serum NOX4 ($p < 0.0001$) and iron ($p = 0.013$) concentrations were found in the patients than in controls. No correlation was found between NOXs, CRP, WBC, ESR and iron metabolism biomarkers in patients.

Conclusion: Our data suggest that increased NOX5 expression and decreased levels of NOX4 might be related with oxidative stress related vascular changes in MS patients. These findings provide future opportunities to combat MS with separately target individual NOX isoforms.

1. Introduction

Multiple sclerosis (MS) is an inflammatory and neurodegenerative disorder. Relapsing-remitting MS (RRMS) is the most common MS pattern characterized by relapses or exacerbations [1]. Although the etiology of the disease is still unknown, it has been proposed that reactive oxygen species (ROS), which are small, oxygen-derived molecules, contribute to MS pathology [2]. Elevated nicotinamide adenine dinucleotide phosphate (NADPH) oxidases activations and concentrations are one of the main enzymatic source of ROS including superoxide anion ($O_2^{\bullet-}$) and its derivatives [3]. The NADPH oxidases (NOX) family consists of seven catalytic homologues. Four NOX isoforms including NOX1, NOX2, NOX4 and NOX5 are expressed from endothelial cells [4].

Blood BBB disruption and vascular changes are determined as one of the prominent and early components in the pathophysiology of MS [5]. Increased expressions of NOX1, 2, and 5 have been related with endothelial dysfunction and vascular inflammation. However, NOX4 exerts protective effects on the vessel wall [4]. There has been growing evidence implicating the role of NOX isoforms in the pathogenesis of several neurodegenerative diseases including Amyotrophic lateral sclerosis, Alzheimer's and Parkinson's disease [6]. However, little is known about the status of serum NOX1, NOX4 and NOX5 in RRMS patients.

Several previous studies have focused on the interaction between MS and nutritional intake to reduce the symptoms such as decreased cognitive, sensory and physical functions [7,8]. One of the important parts of the diet component are micronutrients such as trace elements [9,10]. Abnormal iron depositions have been associated with the production of ROS [11]. Alterations in iron deposition and serum biomarkers of iron metabolism have been consistently reported in patients with MS [12–15].However, uncertainty still exists about the relationship between iron metabolism and oxidative stress in MS.

The aims of this study were to investigate the status of serum endothelial NOXs and its relationship with iron metabolism biomarkers in RRMS patients. Accordingly, serum concentrations of NOX1, NOX4 and

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Table 1
Clinical and demographic characteristics of patients and controls.

Characteristics	Patients	Controls
N	53	45
Male, n (%)	11 (20.75%)	10 (22.22%)
Age	37 ± 10	35 ± 10
Disease duration (years, min - max)	1 – 21 years	N/A
Relaps (yes/no)	10/43	N/A
EDSS (mean ± SD)	2.52 ± 1.47	N/A
EDSS range	1-6	N/A
Interferon beta 1-alpha (n)	20	N/A
Fingolimod HCL (n)	5	N/A
Dimethyl fumarate (n)	3	N/A
Interferon beta 1-beta (n)	11	N/A
Glatiramer acetate (n)	8	N/A
Teriflunomide (n)	4	N/A
Mitoksantron (n)	1	N/A
Natalizumab (n)	1	N/A

N/A: not applicable EDSS: Expanded disability status scale.

NOX5, iron, total iron binding capacity (TIBC) and ferritin were examined in RRMS patients and healthy controls. It is thought that this study provides an exciting opportunity to advance existing knowledge regarding the relationship between oxidative stress and iron metabolism in RRMS patients.

2. Material and methods

2.1. Patients and controls

This prospective study was conducted in the Department of Clinical Biochemistry, School of Medicine, Cumhuriyet University. A total of 98 subjects including 53 RRMS patients and 45 healthy controls were enrolled the study. The mean ages of patients and controls were 37 ± 10 and 35 ± 10 years, respectively ($p > 0.05$). The female to male ratios were 4.8:1 and 4.5:1 in patient and control groups, respectively ($p > 0.05$). Relapse rate was 2.33 in patients. The clinical characteristics of the patients and control subjects are presented in Table 1. The study included patients with clinically diagnosed RRMS according to the 2010 revised McDonald criteria. A relapse was defined as patient-reported symptoms or objectively observed signs typical of an acute inflammatory demyelinating event in the central nervous system (CNS) with a duration of at least 24 h in the absence of fever or infection [16]. The neurological deficit was scored with the Kurtzke expanded disability status scale (EDSS) during the time of disease relapse [17].There were no newly diagnosed patients in the study population. For the healthy control group, the exclusion criteria included a clinical suspicion of any neurological disorders, infections, or the presence of liver disease, kidney disease, rheumatic disease, malignancy, pregnancy, or smoking. To evaluate the nutritional status of the patients' mini nutritional assessment (MNA®) test were performed. Related test can be found following web sites; https://www.mna-elderly.com/forms/MNA_english.pdf. The procedures were approved by the Ethics Committee of Cumhuriyet University in accordance with the ethical standards established by the institution where the experiments were performed or in accordance with the Helsinki Declaration. All participants provided informed consent prior to inclusion in the study.

2.2. Samples and biochemical analysis

Fasting blood samples were collected and sera fractions were separated by centrifugation (3500 rpm, 15 min, and 4 °C). They were then aliquoted and rapidly stored at −80 °C (WiseCryo, South Korea). The quantitative sandwich ELISA technique was used for the determination of serum NOX1, NOX4 and NOX5 (Elabscience Biotechnology Co., Ltd., China). Tests were performed according to the manufacturer's recommendations. Serum ferritin levels were determined with the electrochemiluminescent immunoassay (Roche Cobas e601, Germany). Serum albumin, total protein, iron and unsaturated iron binding capacity (UIBC) concentrations were measured colorimetric method (Mindray BS 2000, China). Total iron binding capacity (TIBC), a marker that measures the blood capacity to bind iron, values were calculated as follow; TIBC: serum iron + serum UIBC. White blood cell (WBC) analysis was performed using autoanalyser (Mindray BC5800, China). C-reactive protein (CRP) measurements were performed using nephelometric method (IMMAGE 800, Beckman Coulter, USA). Erythrocyte sedimentation rate (ESR) was measured Westergreen method (Sistat ESR 100, Ankara, Turkey).

2.3. Statistical analysis

Conformity of the data to normal distribution was evaluated using a histogram, q-q graphs and the D'Agostino & Pearson normality test. The Mann-Whitney U test was performed for NOX1, NOX4, NOX5, ferritin, iron, TIBC, CRP, WBC and ESR for between group comparisons. The correlation between quantitative data was assessed using the Spearman test. Chi-square analysis was used to compare the differences of categorical variables. Data analysis was conducted using R software (https://www.r-project.org/). A value of $p < 0.05$ was considered statistically significant.

3. Results

Mean body mass index (BMI) of the patients were 24.5 ± 4. BMI index values were calculated as follow; patient weight in kg/(height in m^2). Mean total protein and albumin levels were 7.70 ± 0.56 mg/dL and 4.46 ± 0.27 mg/dL in patients, respectively. No patients had abnormal protein (reference range: 6.6–8.3 mg/dL) and albumin (reference range: 3.5–5.3 mg/dL) levels. According to test results, 47 patients had normal nutritional status and 6 patients had risk for malnutrition. Nutritional assessment tests results of the patients were given in Table 2.

Higher NOX5 ($p < 0.0001$) and CRP ($p = 0.014$) concentrations were found in patients than in controls. NOX4 concentrations were lower in patients than in controls ($p < 0.0001$). Box plots for serum NOX4, NOX5 and CRP were given in Figs. 1–3, respectively. No statistically significant difference was observed between patients and controls in terms of NOX1, WBC and ESR. Comparisons of the serum NOX1, NOX4, NOX5, CRP, WBC and ESR values between patients and controls were given in Table 3. It is not found any statistically

Table 2
Mini nutritional assessment test results in patients.

Questions	Yes (n)	No (n)
Has food intake declined over the past 3 months due to loss of appetite, digestive problems, chewing or swallowing difficulties?	3	50
Has suffered psychological stress or acute disease in the past 3 months?	3	50
	# of patients	
Weight loss during the last 3 months		
• Weight loss greater than 3kg	2	
• Weight loss between 1 and 3kg	2	
• No weight loss	49	
Mobility		
• Bed or chair bound		
• Able to get out of bed / chair but does not go out	2	
• Goes out	51	
Psychological problems	0	
• Severe dementia or depression	1	
• Mild dementia	0	
• No psychological problems	52	

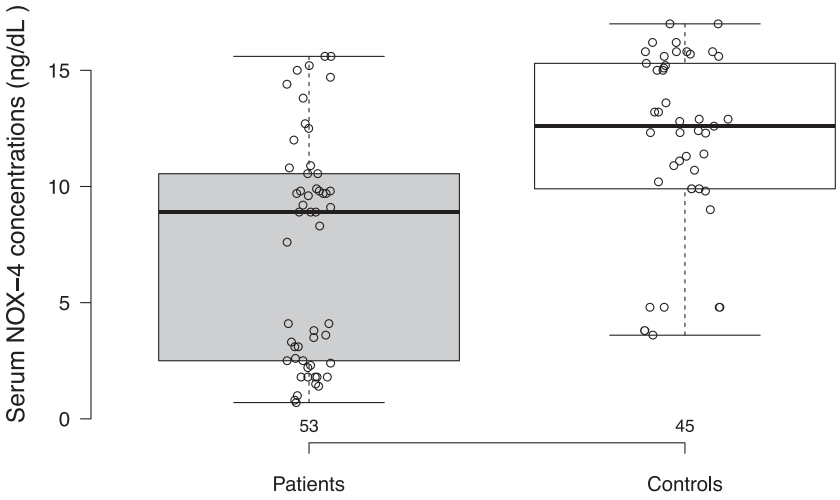

Fig. 1. Box plot of nicotinamide adenine dinucleotide phosphate oxidases 4 concentrations.

significant correlation among CRP, WBC, ESR and studied NOXs levels.

To evaluate the change of ferritin concentrations and the relationship between NOXs, the patients (n = 19) with low iron concentrations were excluded because abnormal concentrations of iron effects serum ferritin concentrations. Therefore, 40–170 μg/dL was used as the reference range for iron. Median ferritin concentrations were 57.00 (16.60–114.50) and 31.00 (20.61–54.32) ng/mL in patients with normal serum iron concentrations (n:34) and the control group, respectively. A statistically significant difference was determined between the patient and control groups in terms of ferritin concentrations (p = 0.040). Lower iron and TIBC concentrations were observed in the patients than in the controls (Table 4). No correlation was determined among serum iron metabolism biomarkers, EDSS, disease duration and NOXs in RRMS patients.

4. Discussion

The main results of the present study were that low serum NOX4 and high serum NOX5, ferritin and CRP levels in patients group. Low iron levels were determined in patient group. No correlations were found among any biochemical parameters in RRMS patients.

The NOXs plays various roles in the host defense, inflammation, post-translational modifications of proteins and neuronal apoptosis in the different parts of central nervous system [18]. Previous studies demonstrated the role of different NOX isoforms in neurodegenerative diseases including Alzheimer and Parkinson's disease [19,20]. Upregulated NOX expressions have been also associated with MS pathogenesis in the experimental and clinical studies [21–24].

Under physiological conditions NOX5 are released at low levels [4]. In the present study, serum NOX5 concentrations were found to be higher in RRMS patients than healthy controls. Compared to other endothelial NOXs very little is known about the NOX5 status in the disease states. NOX5 is responsible for the production of $O_2^{\bullet-}$ and its derivatives [25,26]. Thus, it is thought that elevated NOX5 expressions responsible for the increased oxidative stress in RRMS patients. The increased concentration of some antioxidant enzymes related with the concentration of $O_2^{\bullet-}$ and its derivatives have been reported in MS patients [27,28]. These findings in the previous studies support the driven conclusion.

The lower serum NOX4 levels were found in patients than in controls. In contrast to other endothelial NOXs, endogenous vascular NOX4 has a protective effect on vascular function by increasing nitric oxide bioavailability and suppressing cell death pathways [4,29,30]. Vascular changes and BBB disruption are considered as the important factors in the pathophysiology of MS [5]. Seo et al. showed the association between increased NOXs expression and BBB disruption in the experimental autoimmune encephalomyelitis that is most commonly experimental model for MS [24]. Therefore, it can be considered that downregulated expressions of NOX4 levels contribute to vascular pathology and BBB disruption by inhibiting in RRMS patients.

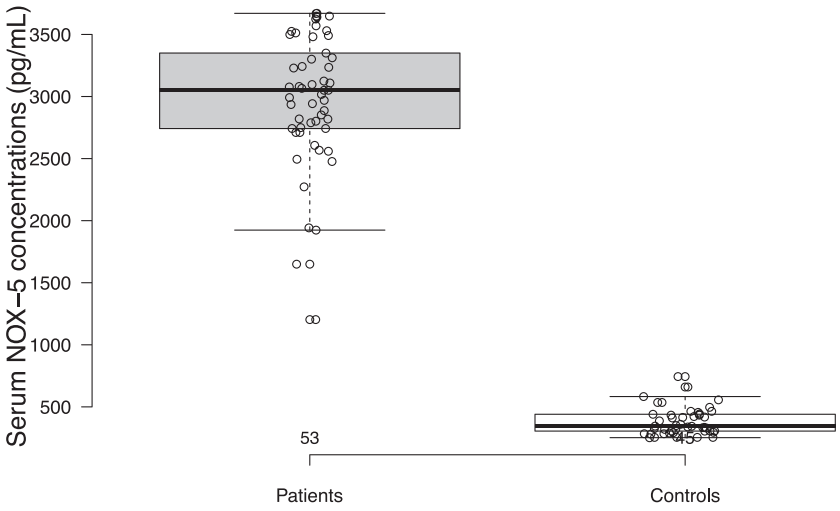

Fig. 2. Box plot of nicotinamide adenine dinucleotide phosphate oxidases 5 concentrations.

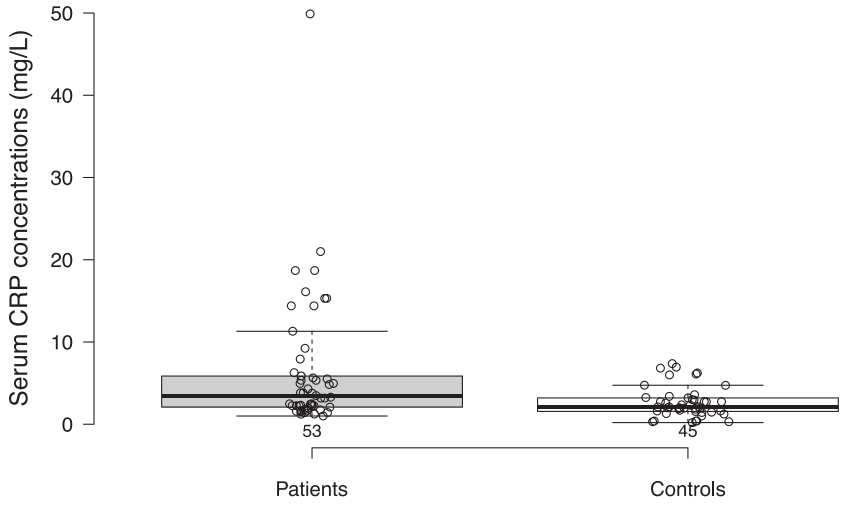

Fig. 3. Box plot of C-reactive protein concentrations.

Table 3
Comparisons of the serum NOX-1, NOX-4 and NOX-5 levels between patients and controls.

Parameters	Patients (n:53)	Controls (n:45)	*P*
NOX-1 (ng/mL)	9.75 (9.50 – 25.95)	9.85 (9.57 – 29.03)	> 0.05
NOX-4 (ng/mL)	8.9 (2.5 – 10.55)	12.6 (9.9 – 15.45)	< 0.0001
NOX-5 (pg/mL)	3050 (2742 – 3416)	343.20 (298.20 – 441.90)	< 0.0001
CRP (mg/L)	3.48 (1.78 – 7.09)	2.10 (1.43 – 3.23)	= 0.014
WBC (10^3/μL)	6.09 (5.00 – 8.58)	7.45 (6.23 – 9.22)	> 0.05
ESR (mm/hour)	11.00 (5.00 – 24.00)	7.50 (5.00 – 10.00)	> 0.05

NOX-1: Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases-1, NOX-4: NADPH oxidases-4, NOX-5: NADPH oxidases-5. CRP: C-reactive protein, WBC: White blood cell, ESR: Erythrocyte sedimentation rate. Results are expressed as median (1^{th}-3^{th} quartiles) with 95% confidence intervals.

Table 4
Comparisons of serum Fe and TIBC concentrations in study population.

Laboratory parameters	Patients (n: 53)	Controls (n: 45)	*P*
TIBC (μg/dL)	332.00 (281.00 – 381.00)	367.00 (313.50 – 425.00)	0.047
Iron (μg/dL)	53.50 (29.75 – 81.00)	81.00 (50.00 – 94.75)	0.013

TIBC: Total iron binding capacity. Results are expressed as median (1^{th}-3^{th} quartiles) within 95% confidence interval.

In the current study, no statistically significant correlation among serum iron biomarkers, NOX1, NOX4 and NOX5 levels is observed. Yauger et al. have reported that the inhibition of NOXs reduces ROS production in the microglia exposed to iron at the in-vitro condition [31]. This contradicting result may be due to the differences of experimental models between studies. Protein-protein interaction, inflammatory signaling and calcium dependent mechanisms are associated with the activation of NOX5. It is proposed that the calcium dependent pathway is the major mechanism [32]. In the recent report, calcium influx through plasma membrane disruption and endoplasmic reticulum degeneration have been associated with axon degeneration and survival in the mice model of MS [33]. Another study reported the increased intracellular calcium in lymphocyte of MS patients [34]. Accordingly, it is thought that the mechanism in the activation of NOX5 might be associated with increased cytosolic calcium level due to the inflammatory degeneration of endoplasmic reticulum and plasma membrane in RRMS patients. It has been reported that the activation of NOX4 is negatively regulated by hydrogen peroxide [35]. It is well known fact that NOX5 increase production of hydrogen peroxide [25,26]. Thus, we thought that increased hydrogen peroxide production due to high NOX5 levels negatively regulate the NOX4 production.

We found lower iron levels in the patients than in controls. Previous studies have revealed that iron insufficiency may play role in the MS pathogenesis and disease progression [36,37]. However, conflicting results have been reported on the concentrations of serum iron metabolism biomarkers in MS [12–15]. Van Rensburg et al. found lower serum iron concentrations in female MS patients than in matched control subjects [15]. While no differences have been reported between control groups and MS patients in terms of serum iron concentrations [12–14]. It is thought that the discrepancy in the results of serum iron concentrations might be related with the differences in the nutritional status of patients. Iron is essential for a variety of enzymes involved in maintaining the health and development of oligodendrocytes, which are the predominant cells in the myelination and remyelination process [38]. Decreased availability of iron in the diet has been associated with hypomyelination [39]. In the current study, only 6 of 53 patients had risk for malnutrition. Thus, it is thought that the other factors including biochemical and genetic should take into consideration for iron status of MS patients as well as nutritional status and serum iron concentrations should be monitored in RRMS patients regardless of whether they are the risk of malnutrition.

Higher ferritin concentrations were determined in RRMS patients with normal iron concentrations compared to healthy controls in the current study. Oliveira et al. were observed elevated serum ferritin concentrations in MS patients compared to healthy controls [40]. However, a recent report has shown no differences between MS patients and healthy controls in terms of ferritin levels [41]. Discordant ferritin results might be due to differences of iron status and neuroinflammation degree of patients between studies. The higher CRP level, a well-known positive acute phase reactants, is observed in MS patients than in controls. It is thought that both increased ferritin and CRP levels could be related to the acute phase reactions owing to the increased CNS inflammation in patients.

5. Conclusion

Our study demonstrated the changes of the serum NOX4 and NOX5 concentrations in RRMS patients. These findings provide evidence for the role of NOX4 and NOX5 enzymes in patients with RRMS. Increased NOX5 expression and decreased levels of NOX4 might be related with vascular damage and BBB disruption in MS patients. These findings provide future opportunities to combat MS with separately target individual NOX isoforms. However, more research on this topic needs to be undertaken to have better understanding the association between

endothelial NOXs and RRMS. We also demonstrated that lower concentrations of iron and TIBC in RRMS patients. Because of the importance of iron on myelination and oligodendrocytes functions serum iron levels should be closely monitored in MS patients.

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Competing interests

None declared.

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