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Oxidative Stress, Serum Mineral and Trace Element Levels in Patients with Multiple Sclerosis with or without Restless Legs Syndrome

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ABSTRACT

Multiple sclerosis (MS) is a central nervous system disease characterized by inflammation, axonal demyelination and multifocal plaques. Sleep problems due to restless legs syndrome (RLS) are frequently encountered in MS patients. Changes in oxidative stress level and liver enzyme activity and deterioration of trace element homeostasis are observed in MS and RLS patients. This study was conducted to determine serum oxidative stress parameters and trace element levels in MS patients with and without RLS.

Thirty healthy individuals (control), 30 MS patients without RLS (MS group) and 30 MS patients with RLS (MS+RLS group) were included in the study. Serum oxidative stress parameters (total antioxidant and oxidant capacities, myeloperoxidase and catalase activities), some liver enzymes (malate dehydrogenase and isocitrate dehydrogenase) activities, blood trace element (copper, zinc and iron) and mineral (calcium, chlorine, magnesium, sodium, potassium) levels were measured.

There was no statistically significant difference between the groups in terms of oxidative stress parameters and liver enzymes. Serum chlorine, potassium and sodium levels were higher in the MS+RLS group compared to the controls ($p<0.001$, $p<0.01$, $p<0.001$, respectively), and the zinc level was lower than the control group ($p<0.05$). Sodium and chlorine levels were higher in the MS+RLS group than in the MS group ($p<0.01$, $p<0.05$, respectively).

Based on these results, varying trace element levels may have value for early prediction of RLS in MS. In studies with larger sample sizes, changing element and liver enzyme activities in the context of oxidative stress may provide clearer information about the diagnosis of RLS.

Keywords: Minerals, multiple sclerosis, oxidative stress, restless legs syndrome (RLS), trace elements

Introduction

Multiple sclerosis (MS) is observed in approximately 2.5 million people in the world and especially in young adult women. It is a chronic central nervous system (CNS) pathology that occurs with the effect of the immune system, demyelination, inflammation, and damage of axons. Five subtypes of MS such as relapsing-remitting MS (RRMS), primary progressive MS, secondary progressive MS, progressive relapsing MS and relapsing progressive MS have been defined in the clinic (1). Neuropathic pain, muscle weakness, decreased physical activity, and progressive neurological disorders are some of the symptoms of MS (2). In addition to these symptoms, sleep disorders are seen 25% to 54% more in MS patients than in other patients. One of the main reasons for this situation is the disorder of the sleep-wake cycle (3). Restless legs syndrome (RLS) is included in the sleep disorders classification. It occurs in the evening and after, usually with the urge or need to move the legs while at rest. It is a progressive sensorimotor and chronic movement disorder. About 26% of individuals with MS are accompanied by RLS pathology, which worsens MS symptoms. MS suppresses the recognition and diagnosis of RLS

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findings in patients. Therefore, patients cannot describe their RLS complaints.

RRMS is a subtype of MS that continues with acute attacks and remissions. It constitutes approximately 85% of MS subtypes (4). In MS pathology, oxidative damage occurs in DNA, protein, and lipids. The increase in reactive oxygen species (5), which are precursors of oxidative damage, results in damage to monocytes, neutrophils, microglia and oligodendrocyte, and axonal damage. It was reported that changes in trace element values increased the total oxidative capacity (TOC) and the total antioxidant capacity (TAC) (6) independently of each other in patients with MS and RLS (7, 8). The enzymes responsible for ROS detoxification are peroxidases such as superoxide dismutase and catalase (CAT) (9). CAT is an anti-inflammatory enzyme that competes with myeloperoxidase (MPO) by converting hydrogen peroxide (H_2O_2) to H_2O and oxygen (10). It is an important intracellular peroxidase, and its expression increases in MS gray matter astroglia and active demyelinating plaques (11). In addition to these oxidative stress parameter enzymes, rapid inactivation of tricarboxylic acid (TCA) cycling enzymes has been reported during oxidative stress (12). Isocitrate dehydrogenase (IDH) and malate dehydrogenase (MDH) are important TCA enzymes. IDH is stimulated by NAD and ADP and inhibited by NADH. It catalyzes the conversion of NAD^+ to α-ketoglutarate, NADH and CO_2 (13). MDH, catalyzes the reversible interconversion of malate and oxaloacetate using NAD(H) (14).

Excessive amounts or deficiency of trace elements and minerals can damage cellular structures known to cause various pathological events. It has been suggested that Fe, Mn, Cu, Zn, Ca, and Mg play a role in the regulation of oxidative stress (15-17). In addition, RLS is associated with K^+ deficiency as it directly causes muscle spasm and muscle twitching. Some physicians even recommend K^+ supplementation to prevent RLS (18). K^+ channel expression has been found to be increased in the region of demyelination (19-21). Concentration imbalance of Na^+ and Ca^+ ions cause chronic inflammation, irregular myelin sheath formation, demyelination, and neuronal dysfunctions. Zn^{+2} is an essential trace element required for growth, development, and healthy maintenance of life in the organism. It participates in processes such as enzyme activities, DNA, protein synthesis, wound healing, and nerve myelination. Zn^{+2} is required to maintain its functionality related to immune system development and immune response formation. Cu^{+2}, another trace element, plays an active role in processes such as nervous system diseases, cancer, and aging. In addition to these effects, it also affects reactions that result in oxidation of proteins, degradation of DNA and RNA, high ROS production and oxidation. It has been reported that the decrease in Cu^{+2} content is associated with degenerative changes in oxidative stress and ROS-related diseases by causing hydroxyl radical formation (22). In our previous study, we determined the differences in serum trace element levels due to oxidative stress in different neurological diseases (23).

Understanding the mechanisms and facilitating the diagnosis of RLS in patients with RRMS will contribute to earlier management of this problem, preventing its progression and reducing the socioeconomic burden caused by these conditions are gaining importance today. When we reviewed the literature, we found different studies on oxidative stress and trace elements in MS patients. However, we could not find a study comparing oxidative stress, changes in liver enzyme activity levels, trace element levels in MS patients with or without RLS. We wondered about the differences in oxidative stress and trace element levels in patients with MS accompanied by RLS compared to patients without RLS. Therefore, in this study, investigated serum oxidative stress parameters (TOC, TAC, myeloperoxidase, isocitrate dehydrogenase and catalase activities), some liver enzyme activities (malate dehydrogenase and isocitrate dehydrogenase), blood trace elements (copper, iron, and zinc) and minerals (calcium, chlorine, magnesium, sodium and potassium). We compared the results of the analysis statistically in MS patients with and without RLS.

Materials and Methods

This study was designed to compare serum oxidative stress, liver enzymes and TCA enzyme levels in MS patients with and without RLS. Patients who applied to the neurology outpatient clinic between April 2021 and January 2022 were included in the study. Blood serum samples were collected according to inclusion and exclusion criteria. This study conforms to the principles of the Declaration of Helsinki and has been validated by the Bezmialem Vakif University clinical research ethics committee (Ethics number: 54022451/ 2 March 2021). Informed consent form was read and signed by the participants to obtain their consent that they participated the study voluntarily.

Population of Study: In reference to previous studies, power analysis calculated the minimum sample size for each group as 25, with a total of 75 at a 95% confidence level. The control group (n=30) consisted of healthy people between the ages of 18-65 who did not have any disease in their routine examinations. The MS group (n=30) consisted of RRMS patients of the same age and gender as the control group. RRMS was diagnosed according to McDonald's 2017 diagnostic criteria (24). The MS+RLS group (n=30) consisted of patients with RRMS accompanied by RLS. The diagnosis and severity of RLS was determined based on the patient's statement using the modified "Restless Legs Syndrome Severity and Rating Scale" (25). The age, gender and drug use information of the patients were recorded in written form. The collected blood was centrifuged, and the serum was stored at -80°C until the day of analysis.

Inclusion Criteria: People aged between 18-65 years and diagnosed with RRMS according to McDonald's 2017 diagnostic criteria were included in the study.

Exclusion criteria: Pregnant women, individuals with additional neurological diseases (Parkinson's disease, dementia, stroke, polyneuropathy, myasthenia gravis), individuals with diabetes, individuals who had an attack within 3 months and individuals who used any steroid medication within 3 months were not included in the study.

Biochemical Parameters: Serum TAC level was calculated by the colorimetric method developed by Erel (26). The basis of the technique is to measure the amount of OH radical. The iron ion of o-diacidine generates OH radical by Fenton type reaction with H_2O_2 and the colour changes depending on o-diacidine. Serum antioxidants neutralize oxidants and prevent colour change. This technique identifies the antioxidant capacity to counter oxidative free radical reactions initiated by OH.

Serum TOC level was determined by a photometric method developed by Erel (27). The basis of the technique is that serum oxidants oxidize iron ions of o-dianisidine to ferric ions. Ferric ions formed in acidic medium by this oxidation are coloured xylenol orange. The changing colour intensity is related to the presence of oxidants in the serum.

Serum IDH enzyme activity was calculated with the marketed available Rel Assay $NADP^+$ - Dependent Isocitrate Dehydrogenase assay kit (RL 0949, Gaziantep, Turkey) by photometric method (Abbott ARCHITECT c8000 analyzer). The basis of the method is the reaction in which the $NADP^+$ molecule is converted to its reduced form $NADPH^+H^+$ by the IDH enzyme in the presence of the substrate isocitrate. Increasing $NADPH^+H^+$ concentration over time produces a change in absorbance at 340 nm that is directly related to enzymatic activity. IDH enzyme activity is calculated using the molar absorption coefficient of $NADPH^+H^+$ ($6.3 \times 103\ M^{-1}\ cm^{-1}$).

Serum MDH enzyme activity was calculated with the marketed available Rel Assay Malate Dehydrogenase measurement kit (RL 0925, Gaziantep, Turkey) by photometric method (Abbott ARCHITECT c8000 analyzer). The basis of the method depends on the reaction in which the $NADH^+H^+$ molecule is converted to its oxidized form, NAD^+, by the MDH enzyme in the presence of the substrate oxaloacetate. The subsequent decreasing $NADH^+H^+$ concentration produces a change in absorbance at 340 nm which is directly related to the enzymatic activity. MDH enzyme activity is calculated using the molar absorption coefficient of $NADH^+H^+$ ($6.3 \times 103\ M^{-1}\ cm^{-1}$).

Serum MPO enzyme activity was calculated method developed by Bradley et al. (28), The basis of the technique is based on the kinetic measurement of the rate of formation of the coloured product of the oxidation of odianisidine with MPO in the presence of H_2O_2 at 460 nm. MPO activity was expressed as units per liter of serum (IU/L).

Serum CAT enzyme activity was made at 410 nm by the Aebi method (29) based on the breakdown of H_2O_2 (U/ml). The basis of the technique is that the enzyme catalase converts H_2O_2 into water and oxygen. CAT enzyme activity reduces the presence of H_2O_2 as a result of the enzyme's dismutase reaction in an environment containing H_2O_2. Horseradish Peroxidase (HRP) reacts with the substrate in the presence of hydrogen peroxide to convert the colorless substrate to a pink colored product. Increasing catalase presence in the samples results in decreased hydrogen peroxide concentration and reduced pink product. The change in absorbance of the pink color is proportional to catalase activity in the sample.

Serum Zn concentrations were measured with a marketed available Rel Assay zinc measurement kit (Gaziantep, Turkey) using photometric method (Abbott ARCHITECT c8000 clinical chemistry analyzer). The basis of the Zn^{+2} technique is that the total zinc in the sample changes the red-orange color of 5-Br-PAPS to light pink under

alkaline conditions. The change in absorbance measured at 548 nm is proportional to the Zn^{+2} concentration (µg/dL) in the sample. The standard of the method is zinc sulphate.

Serum Cu concentrations were measured with a marketed available Rel Assay copper measurement kit (Gaziantep, Turkey) using photometric method (Abbott ARCHITECT c8000 clinical chemistry analyzer). The basis of the technique is that the red-orange DiBr-PAESA turns purple under alkaline conditions proportional to the copper presence in the samples (µg/dL). The absorbance change is measured at 572 nm and Cu sulphate is used as the standard.

Serum Ca^{+2}, Mg^{+}, Fe^{+2}, K^{+}, Na^{+} and Cl^{-} concentrations were measured with a marketed available routine Abbote measurement kit using Abbott ARCHITECT c8000 clinical chemistry autoanalyzer.

Statistical Analysis: The distribution of data was tested with the Shapiro-Wilk test. Data with normal distribution were analyzed using the one-way ANOVA test. Those that did not fit the normal distribution were tested using the Kruskal-Wallis test. Post-hoc comparisons were made with Dunnet and Dunn tests. Analyses were performed with the program (GraphPad Prism, Version 8.0.1 Software Program, San Diego, CA). Data were calculated as mean ± standard error of mean. $P<0.05$ was set as the statistically significant level.

Results

The disease duration of both groups was found to be close to each other, with an average of 6 years. The RLS severity (19.28±1.19) in MS patients was moderate (Table 1). The distribution of RLS severity among the MS patients was determined as mild 10% (3 people), moderate 53.33% (16 people), severe 26.67% (8 people), and very severe 10% (3 people). Although RLS can range in severity from moderate to severe, only 2 patients were using modafinil and 1 patient was using pregabalin. The rest of the patients were not using sleep-related drugs including levodopa, ropinirole-pramipexol-rotigotine, dopamine agonists, opioids, anticonvulsants or benzodiazepines preferred in RLS.

Regarding to oxidative stress parameters (TAC, TOC, MPO and CAT activities) and some liver enzymes (IDH and MDH) activities, no difference was found among groups (Figures 1-4).

All the serum parameters evaluated were within the reference range in the three groups studied. Serum Cl^{-}, K^{+} and Na^{+} levels were significantly higher ($p<0.001$, $p<0.01$, $p<0.001$, respectively, Table 2) and zinc ($p<0.05$) levels were lower in the MS+RLS group compared to the controls (Table 3). Na^{+} and Cl^{-} levels were higher in the MS+RLS group than in the MS group ($p<0.01$, $p<0.05$, respectively, Table 2).

Twenty-seven MS patients without RLS and 26 MS patients with RLS were using immunomodulatory drug therapy. While none of the patients without RLS used symptomatic medication, 7 patients in the MS group with RLS were using additional symptomatic medication (Table 4).

Discussion

The relationship and mechanisms of sleep disorders with neurological disorders attract attention today. Sleep disturbance is more common in MS patients than in healthy people. In a broad-based cross-sectional clinical study with 1063 patients, mild sleep problems were reported in 13.3%, moderate in 21.5%, and severe in 30% of the patients (3). In a study conducted in the French adult people, the incidence of RLS was found to be 18% in 242 MS patients. It was determined to have a more widespread distribution and severity in the MS group (30). In our study, the RLS severity (19.28±1.19) in MS patients was moderate. The distribution of RLS severity among the MS patients was determined as mild 10%, moderate 53.33%, severe 26.67% and very severe 10%.

Oxidative Stress Parameters: TAC, TOC, Catalase and Myeloperoxidase: Recent studies have measured TAC and TOC levels to compare inflammation status in patients with MS (31, 32). TAC measurement enables the determination of the status of antioxidants (33). In a study with cerebrospinal fluid and blood serum samples, TAC level was found to show high potential for diagnosis of MS disease state (34). However, in another study, when the serum TAC and TOC levels of 22 RLS patients were examined, no statistical difference was found between the RLS group and the controls (35). The results of our study were inconsistent with the results of the Khajenobar et al, but were consistent with the finding of Cikrikcioglu et al. In a study conducted with the blood serum of Japanese MS patients, it was reported that MPO expression was significantly higher than the control group (36). In a study conducted in the Netherlands on different types of MS, mean leukocyte MPO expression was found lower in RRMS patients than in

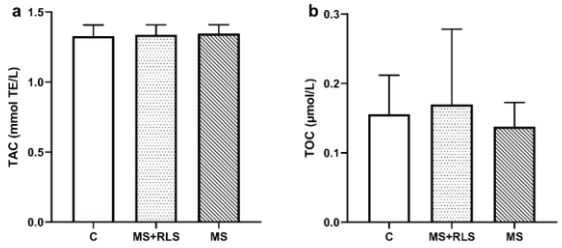

Fig. 1 Serum TAC (a) and TOC (b) values of the study groups (n=30)
TAC, total antioxidant capacity; TOC, total oxidant capacity; C, control; MS, multiple sclerosis; MS+RLS, multiple sclerosis with restless legs syndrome. No difference was found among groups

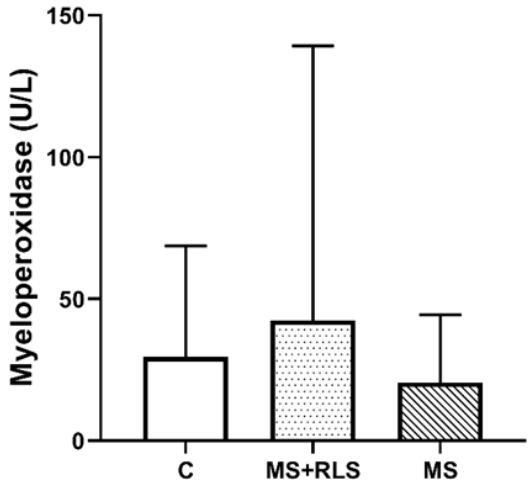

Fig. 2 Serum myeloperoxidase activities of the study groups (n=30)
C, control; MS, multiple sclerosis; MS+RLS, multiple sclerosis with restless legs syndrome. No difference was found among groups

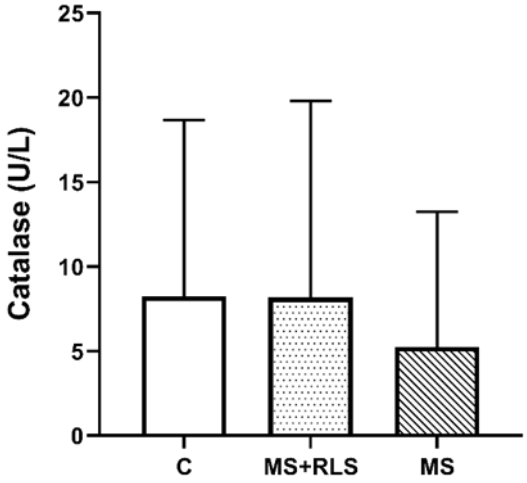

Fig. 3 Serum catalase activities of study groups (n=30)
C, control; MS, multiple sclerosis; MS+RLS, multiple sclerosis with restless legs syndrome. No difference was found among groups

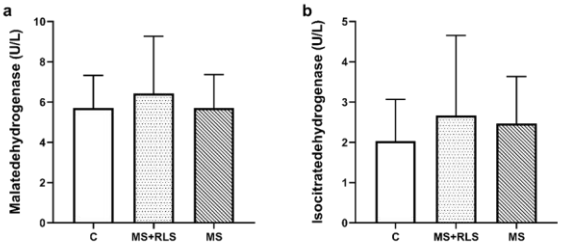

Fig. 4 Serum malate dehydrogenase (a) and isocitrate dehydrogenase (b) activities of the study groups (n=30)
C, control; MS, multiple sclerosis; MS+RLS, multiple sclerosis with restless legs syndrome. No difference was found among groups

controls (37). In our study, regarding to MPO activities, no difference was found among groups. The previous findings had indicated that lymphocytic catalase activity remained unchanged, while in granulocytes of MS patients, it decreased by 50% in comparison to the normal control group (38). Catalase expression had been found to be high in gray matter astroglia and active demyelinating plaques obtained from brain tissues of individuals with MS (39). However, there were no statistical differences in catalase activity levels among the groups investigated in our study.

Glucose Metabolizing Enzymes: IDH and MDH: Metabolic dysregulation has been assumed to play a role in the pathophysiology of various neurodegenerative diseases like Alzheimer's disease, Huntington's disease, and Parkinson's disease. Whilst the exact etiology of Multiple Sclerosis (MS) remains incompletely elucidated, it has been suggested that there may exist analogous alterations in the disease process, as several other neurodegenerative disorders have been observed to disrupt cerebral energy metabolism (40). In an animal study with an MS model constructed on this idea, it had been reported that IDH expression decreased in the CNS with hypoxia-like tissue injury (41). In our study, regarding to serum IDH activities, no difference was observed between patient and control groups. The utilization of a highly sensitive methodology, such as proteomics, and conducting with tissues instead of serum causing with death of animals, may account for the discrepancies observed between the findings of these investigations.

In a study with plaque tissues in humans with MS, MDH was reported to be increased in active MS plaques of patients (42). It was also reported that the level of MDH was also decreased in CSF of MS patients compared to the control groups (43). In relation to these findings, although the MDH

Table 1: Age, gender and RLS severity of the study groups (n=30)

PARAMETERS	C	MS+RLS	MS
Age	32.30±2.42	40.93±2.19	35.93±1.92
Gender	22F, 8M	24F, 6M	24F, 6M
RLS severity	0	19.28±1.19	0
Number of drug users	0	27	27

C, control; MS, multiple sclerosis; MS+RLS, multiple sclerosis with restless legs syndrome; F, female; M, male. Data are expressed as mean ± standard error of means

Table 2: Serum mineral levels of the groups (n=30)

PARAMETERS	C	MS+RLS	MS	RR
Ca (mg/dl)	9.43±0.08	9.59±0.08	9.55±0.07	8.8-10.6
Cl (mmol/l)	101.7±0.43	104.4±0.42***+	102.6±0.46	98-106
K (mmol/l)	4.43±0.09	4.72±0.08**	4.56±0.07	3.5-5.1
Mg (mg/dl)	1.85±0.03	1.85±0.02	1.82±0.02	1.8-2.6
Na (mmol/l)	139.4±0.35	141.2±0.45***++	138.7±0.55	136-146

C, control; MS, multiple sclerosis; MS+RLS, multiple sclerosis with restless legs syndrome; RR, reference range. Data are expressed as mean ± standard error of means. ** $p<0.01$, *** $p<0.001$ statistically significant compared to the control group. $^{+}$ $p<0.05$, $^{++}$ $p<0.01$ statistically significant compared MS+RLS with the MS group

Table 3: Serum trace element levels of the groups (n=30)

PARAMETERS	CONTROL	MS+RLS	MS	RR
Cu (µg/dl)	110.3±5.08	112.4±6.89	110.3±3.39	70-165
Fe (µg/dl)	80.83±8.51	83.43±8.11	79.97±5.52	60-180
Zn (µg/dl)	95.17±2.20	88.63±1.85*	91.07±1.95	66-110

MS, multiple sclerosis; MS+RLS, multiple sclerosis with restless legs syndrome; RR, reference range. Data are expressed as mean ± standard error of means. * $p<0.05$ statistically significant compared to the control group

Table 4: Drugs used in patient (n=30)

MEDICINES	C	MS+RLS	MS
Immunomodulator			
Medicines	0	26	27
IFN-beta		5	2
Glatiramer acetate		4	6
Teriflunomide		6	3
Fingolimod		9	7
Natalizumab		0	2
Ocrelizumab		0	2
Dimethyl fumarate		2	5
Symptomatic Drugs	0	7	0
Modafinil		2	0
Fampiridin		1	0
Baklofen		2	0
Gabapentin		1	0
Pregabalin		1	0

C, control; MS, multiple sclerosis; MS+RLS, multiple sclerosis with restless legs syndrome

activity level was high in the MS+RLS group in present study, there was no significant difference was found between the groups.

Trace elements: Copper (Cu) and Zinc (Zn): Chen and colleagues observed a significant elevation in serum Zn^{+2} levels in patients with Restless Leg Syndrome (RLS) compared to healthy controls, but no marked variation in Cu^{+2} levels (44). Conversely, Jiménez et al. aimed to corroborate these findings in a cohort of 100 RLS patients, but reported a non-significant decrease in Zn^{+2} values (45). Consistent with this investigation, the MS+RLS cohort exhibited a statistically significant reduction in serum Zn^{+2} levels relative to the control group ($p<0.05$), while no significant alteration in Cu^{+2} levels was observed. Previous studies have documented an upregulation of ZIP8, a zinc transporter protein on the erythrocyte cell membrane, in conditions that are associated with heightened oxidative stress, such as RLS (46). The decreased levels of Zn observed in the patient group may be linked to this phenomenon, whereas the absence of a significant difference between groups in terms of their oxidative stress parameters may be attributed to the limited sample size (n=30). Previous research has identified the co-occurrence of Fe deficiency and chronic inflammation in patients with MS and RLS. Secondary RLS subtypes, such as iron deficiency anemia and pregnancy-related RLS, have been documented in this population, suggesting that these conditions may contribute to the development of RLS in MS (47, 48). Nonetheless, our investigation did not reveal any significant differences in terms of iron levels between the MS+RLS group and the control group. The effects of potassium ion release on microglia-induced remyelination capacity following experimental demyelination were assessed (49). In a study conducted with MS patients, serum potassium levels were found to be significantly lower depending on lower intake with diet in MS patients (50). In our study, serum K^+ levels were found to be statistically higher in MS+RLS group compared to controls ($p<0.01$). Studies have demonstrated that individuals with multiple sclerosis (MS) exhibit heightened expression of the voltage-gated potassium channel Kv1.3 on the membrane of peripheral T cells (51). Given that potassium channel inhibitor medications have been shown to increase serum potassium levels in MS patients with restless leg syndrome (RLS), the observed elevation of potassium levels in this patient population may be attributed to the use of such pharmacological agents. It has been reported that intracellular and extracellular sodium concentration tends to increase due to the increase in MS severity in MS patients (52). Depending on the severity of hypernatremia, it causes central nervous system dysfunction and damage (53). In our study, serum Na^+ level was found to be statistically higher in MS+RLS group compared to controls ($p<0.001$). The data of our study supports the literature results. Na^+ level was found to be statistically significantly higher in the RLS+MS group compared to the MS group ($p<0.01$). This may be the hallmark of MS and the RLS pathology accompanying MS. Excessive sodium intake has been associated with increased clinical and radiological findings in MS patients with disease severity (54). In a study conducted with MS patients, it was shown that serum chlorine levels were significantly lower than controls (55). Serum Cl^- values of MS+RLS patients participating our study were found to be higher than controls. Cl^- level was found to be statistically significantly higher in the RLS+MS group compared to the MS group ($p<0.05$). The effect of this anion on RLS and MS may become clear when considered together with Na^+, which showed high significance in our study.

It is a known fact that the change of minerals and trace elements affects oxidative stress. It is also known that the activity levels of glucose metabolizing enzymes are affected when oxidative stress changes. Our study differs from the existing literature in terms of the parameters we examined and compared in MS patients with or without RLS. In this context, we think that it will contribute to the literature on MS and RLS. In particular, it was concluded that the MS+RLS group showed a significant difference in some mineral and trace element levels compared to the control group, and the Na and Cl levels in the MS+RLS group compared to the MS group. Our study has several limitations with small sample size and lack of various methodologies such as genetic and molecular evaluations to investigate mechanisms in more detail.

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