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# Dietary zinc status is associated with ZnT3 (SLC30A3), IL-6 gene expressions and spinal cord tissue damage in spinal cord tissue in a cuprizone-induced rat Multiple Sclerosis model

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ABSTRACT

The aim of this study was to investigate the effects of dietary zinc status on spinal cord tissue damage and ZnT3, IL-6 gene expressions in a cuprizone-induced rat Multiple Sclerosis (MS) model. The study was carried out on 46 adult male rats of the genus Wistar. The animals used in the study were divided into 5 groups (G) (Control 6, other groups 10). G1, Control. G2, Sham-MS: Carboxy-methyl-cellulose (KMS) solution in which Cuprizon was dissolved was given to rats by gavage daily for 8 weeks at the rate of 1 % of daily feed consumption. MS was formed by giving 1 % of the daily feed consumption cuprizon in KMS solution by gavage to the animals in G3, 4 and 5 for 8 weeks. G4 was fed with a zinc deficient (50 μg/kg zinc) diet. G5 was given intraperitoneal (ip) zinc sulfate (5 mg/kg/day) supplementation. MS formation in animals was determined by Rotarod tests and Myelin Basic Protein (MBP) gene expression analysis. ZNT3 and IL-6 gene expression levels in spinal cord tissue samples of animals by Real-Time-PCR method; MDA and GSH levels were determined by ELISA method. The highest spinal cord MDA and IL-6 levels were obtained in G3 and G4 ($P<0.05$). Zinc supplementation in G5 prevented the increase in the mentioned parameters and turned them into control values ($P<0.05$). The spinal cord GSH and ZnT3 levels of G3 and G4 were lower than all other groups ($P<0.05$). Zinc supplementation prevented suppression in the same parameters in G5 and reached the control values ($P<0.05$). The findings of the current study suggest that zinc supplementation in addition to treatment for MS may be beneficial in reducing the severity of the disease.

## 1. Introduction

Multiple sclerosis (MS) is a neurodegenerative disorder that causes toxicity in different organs, such as spinal cord tissue. Although the brain has traditionally been considered the main tissue involved in MS, the exact etiology remains unknown. The heterogeneous clinical symptoms of MS, mainly visual and sensory motor, are associated with lesion of the spinal cord and peripheral nervous system. Spinal cord demyelination and atrophy are common in MS and are easier to detect clinically [1]. The MS animal model, experimental autoimmuneencephalomyelitis (EAE), is widely used for studies of human inflammatory demyelinating diseases [2]. The most frequently used of these models is the Cuprizon model [3]. The cuprizone model is a toxic demyelination model. In this model, young mature mice are fed cuprizone [4]. This causes permanent demyelination [3,4]. Remyelination begins to occur spontaneously 4 days after discontinuation of the neurotoxin, and therefore the cuprizone model is a good study model to ensure demyelination and observe remyelination [5]. Demyelination formation with cuprizone is a preferred experimental model because it is a model that can be easily monitored and does not damage any cells except oligodendrocytes [3,5].

Zinc is an essential micronutrient abundant in the brain with various roles in development, injury, and neurological diseases [6,7]. There is increasing evidence that disorders in zinc regulation occur in patients

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with MS [8]. It has already been recently reported that circulating zinc levels decrease in MS patients [9]. The idea that zinc plays an important role in the pathogenesis of MS arises from its high concentration in the central nervous system and its participation in brain physiology [8]. Diseases such as acute brain injury and neurodegenerative diseases are accompanied by impaired zinc regulation [10]. Zinc transporter proteins (ZnTs) play a critical role in regulating zinc distribution in the central nervous system [11]. It has been reported that ZnT3 gene deletion, one of these carrier proteins, greatly reduces the daily clinical score in the experimental MS model by suppressing inflammation and demyelination in the spinal cord [11,12]. It has also been reported that ZnT3gene deletion significantly inhibits the formation of abnormal synaptic zinc patches associated with multiple sclerosis and disruption of the blood-brain barrier [8]. These two studies strongly support the idea that ZnT3-mediated zinc release from presynaptic terminals may play a role in the pathogenesis of multiple sclerosis. Additionally, metallothioneins (MT) are a family of intracellular proteins that bind zinc and act as a zinc buffer [8]. Since metallothioneins have been shown to reduce cytokine expression and apoptotic neuron death in an experimental MS model, it is thought that metallothioneins may be a potential treatment target for MS [8].

Increase in the production of reactive oxygen species (ROS) and deterioration in antioxidant parameters in the spinal cord tissue of MS patients accompany the above-mentioned clinical symptoms [1]. It has also been reported that the decrease in metallothionein levels may be associated with spinal cord tissue damage in MS patients [13].

It has been clearly shown that Glutathione peroxidase (GPx), which is already an important antioxidant enzyme, and Glutathione (GSH), which it uses as an electron donor, decrease in the experimental MS model [14]. Interleukin-6 (IL-6) is a soluble, pleiotropic cytokine that plays a key role in many biological processes. It is a primary regulator of acute and chronic inflammation, contributing to the initiation and/or maintenance of various autoimmune and inflammatory disorders. The reported increase in IL-6 levels in MS patients is in line with the above information [15]. It has been shown that zinc treatment, an antioxidant element, reduces IL-6 levels, which increase in various pathological processes, and may prevent tissue damage by causing GSH activation [16]. The aim of this study is to investigate the effects of zinc supplementation and dietary zinc deficiency on spinal cord tissue damage in the Cuprizone-induced rat Multiple Sclerosis model.

## 2. Materials and Methods

The research was carried out on 46 adult male Wistar rats at Selçuk University Experimental Medicine Research and Application Center. The analyzes of the study were carried out in the Molecular Physiology laboratory of Selçuk University Faculty of Medicine, Department of Physiology. The study protocol was approved by the local animal ethics committee (ethics committee approval dated 26.03.2021 and decision number 2021–18).

## 3. Experimental Design

The animals were kept in a room with a 12-hour dark and 12-hour light cycle, where the temperature was 21 $^{0}$C and the humidity was 40–60 % ncm. 40 animals treated with cuprizone and Carboxy-methyl-cellulose (KMS) were kept in a total of 8 cages, with 5 animals in each cage, and 6 animals in the control group were kept in 1 cage. Throughout the study, diet and treatment interventions on the rats were carried out in accordance with international standards, and unnecessary harm to the animals was prevented at all costs (Table 1), [3].

Experimental animals were divided into 5 groups in total.

Group 1, Control: No treatment was applied to the animals in this group.

Group 2, Sham-Multiple Sclerosis: Carboxy-methyl-cellulose solution, in which cuprizone would be dissolved, was prepared with pure water at a concentration of 1 % for the rats in this group. The prepared solution was given via gavage at a rate of 1 % (0.66 ± 6 g/kg) of daily feed consumption. Applications were made daily for 8 weeks.

Group 3, Multiple Sclerosis: MS was induced by gavage of cuprizone in KMS solution at the rate of 1 % of daily feed consumption to the rats in this group for 8 weeks.

Group 4: Multiple Sclerosis-Zinc Deficient Diet: In the rats of this group, MS was induced by gavage of cuprizone in KMS solution at a rate of 1 % of daily feed consumption for 8 weeks. The animals in this group were also fed with a zinc-deficient diet (50 μg/kg) for 8 weeks until the end of the study [17].

Group 5: Multiple Sclerosis + Zinc Supplemented Diet: In the rats of this group, MS was induced by gavage of cuprizone in KMS solution at a rate of 1 % of daily feed consumption for 8 weeks. Additionally, animals in this group were supplemented with intraperitoneal (i.p.) zinc sulfate (5 mg/kg/day) for 8 weeks until the end of the study [18].

The zinc content in the standard rat feed supplied by Selçuk University Experimental Medicine Research and Application Center was 95.18 mg/kg.

## 4. Experimental applications

### 4.1. Cuprizon application

Bis (cyclohexanone) oxaldihydrazone (Supelco-14690) obtained from Sigma Aldrich was used to create the MS model. To induce MS, cuprizone was given to the animals daily by gavage in KMS at a dose of 0.66 g/kg for 8 weeks. Various doses of cuprizone are recommended in the literature to induce MS in rats. Cuprizone application is made by mixing it into the standard feed of animals at rates of 0.5 %, 1 %, 2 % [19].

### 4.2. Zinc application

After dissolving zinc-sulfate in distilled water, zinc sulfate supplementation at a dose of 5 mg/kg/day in 0.5 ml physiological saline was administered by ip injection. Zinc sulfate application was carried out daily for 8 weeks.

### 4.3. Carboxy methyl cellulose (KMS) application

Carboxy methyl cellulose, which was used to dissolve cuprizone, was prepared as a weekly stock. It was given to the sham group via gavage daily for 8 weeks.

**Table 1**
Experimental Design.

| | GROUP 1 (CONTROL) | GROUP 2 (SHAM-MS) | GROUP 3 (MS) | GROUP 4 (MS- ZINC DEFICIENT) | GROUP 5 (MS- ZINC SUPPLEMENTED) |
|---|---|---|---|---|---|
| ANİMAL NUMBERS | 6 | 10 | 10 | 10 | 10 |
| KMS APPLICATION | | X | X | X | X |
| CUPRIZON APPLICATION | | | X | X | X |
| STANDARD RAT DIET | X | X | X | | X |
| ZINC DEFICIENT DIET | | | | X | |
| ZINC SUPPLEMENT | | | | | X |

### 4.4. Determination of MS formation

MS formation in animals was determined by Rotarod tests and Myelin Basic Protein (MBP) gene expression analysis. These analyzes were used in another study investigating the MS-corpus callosum relationship. For this reason, it was preferred to use these data in this article by citing the source only in the material and method section (Table 2, Figs. 1 and 2) [20].

### 4.5. Obtaining spinal cord tissue

At the end of the eight-week application, spinal cord tissue samples were taken from all animals under general anesthesia. General anesthesia Ketalar 60 mg/kg, "Parke-Davis"; and xylazine 5 mg/kg was achieved by intramuscular administration of the combination "Rompun, Bayer". The spinal cord tissue samples taken were stored at −80 $^{0}$C until analysis.

## 5. Biochemical analysis

### 5.1. Total RNA isolation and cDNA synthesis in spinal cord tissue

Approximately 50 mg of spinal cord tissue was obtained and total RNA was isolated using a commercial kit (Bio Basic, Canada). The quality and quantity of the RNA obtained was determined using the SMA nanodrop device. RNAs with an OD260/280 ratio of 1.9 and above were stored at −20°C for use in the study. The synthesis of complementary DNA (cDNA) was then started.

For cDNA synthesis, a commercial cDNA synthesis kit was used (catolog number: 1708891; Bio-Rad-USA). 5x iScript Reaction Mix (4 µl), iScript Reverse Transcriptase (1 µl), Nuclease-free water (5 µl), Total RNA (25ng-10 µl) were prepared. The cDNAs were obtained using a Thermal Cycler (Biorad, USA).

### 5.2. Real-time qPCR analyses

The cDNA obtained was used to determine the expression level of the ZNT3 and IL-6 genes. All reactions were performed in triplicate using FastStart Essential DNA Green Master Mix (Switzerland) in a volume of 20 µl.

Primers used for expression analysis of target genes at the mRNA level were obtained from the manufacturer (Oligomer, Turkey). GAPDH was used as an endogenous control (Table 3).

Reaction volumes consisted of 10 µl master mix (enzyme, dNTP, Mg, buffer and water), 1 µl cDNA, 1 µl forward and 1 µl reverse primers (10 pmol/µl) and 7 µl RNAse-free water.

After primer optimisation, PCR conditions were as follows: initial denaturation at 95°C for 10 minutes, denaturation at 95°C for 10 seconds, annealing at 60°C for 10 seconds and polymerisation at 70°C for 10 seconds, and the number of cycles was 45. Melting curve analysis was performed as 1 cycle of 15 seconds at 95°C, 10 seconds at 60°C, 10 seconds at 95°C. All reactions were performed on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA).

The data obtained were analysed using the comparative Ct (ΔΔCt) relative quantification method and prepared for comparison between groups [21].

**Table 2**
Rotarod test and Spinal Cord Myelin basic protein (MBP) gene expression levels of the study groups.

| Groups | ROTAROD TEST Remaining time on the stick "seconds" Mean±SD | MBP ($2^{-\Delta\Delta CT}$) Mean±SD |
|---|---|---|
| Control Group (G1) | 209,66±50,06**a** | 0,95±0,13b |
| Sham Group (G2) | 178,50±87,44**a** | 0,98±0,09b |
| MS Group (G3) | 58,60±23,66**c** | 1,58±0,13a |
| Zn deficient MS group (G4) | 35,00±16,61**c** | 1,59±0,09a |
| Zinc supplemented MS Group (G5) | 89,40±50,83**b** | 1,09±0,16b |

a>b>c: The difference between averages with different letters in the same column is important (P<0,05).
**P P-Values MBP**: G1-G2=0990 G1-G3=0000, G1-G4=0000, G1-G5=0260, G2-G3=0000, G2-G4=0000, G2-G5=0368, G3-G4=1000 G3-G5=0000, G4-G5=0000

### 5.3. Determination of Malondialdehyde (MDA) and Glutathione (GSH) levels in spinal cord tissue

In this study, MDA as an indicator of tissue damage and Glutathione (GSH) as an indicator of antioxidant activity were analyzed in spinal cord tissue. Measurements were made on the SPECTROstar (BMG Labtech, Germany) device. MDA levels in spinal cord tissue were determined using the method of Mihara and Uchiyama [22] and the results were defined as nmol/g tissue. GSH analyzes in spinal cord tissue were determined by the Ellmann method [23]. Results were expressed as mg/g/protein.

### 5.4. Statistical evaluations

Statistical evaluation of the findings was made with a computer package program (SPSS 26.0). Arithmetic means and standard deviations of all parameters were calculated. The "Shapiro-Wilk" test was performed to determine the homogeneity of the data and it was determined that the data showed a normal distribution. Arithmetic mean and standard deviation values and IQR values were calculated for all data showing a normal distribution. "One-way analysis of variance (ANOVA)" test was used to determine the difference between the groups. The "Duncan" test, one of the multiple comparison tests, was used to determine which group caused the differences. Differences at P<0.05 were considered significant.

## 6. Results

### 6.1. Weight changes in rats

In our study, at the end of the 8-week application, we obtained the lowest body weight in rats in the MS group (G4) fed with a zinc-deficient diet (P<0.05). The body weights of the MS group (G5) receiving zinc supplements were lower than the sham group (P<0.05) and were not different from the control (G1) and MS (G3) groups (Table 4).

### 6.2. MDA and GSH findings in spinal cord tissue

The highest MDA and lowest GSH values in the spinal cord tissue in our study were obtained in the cuprizone-induced MS group (Group 3) and the cuprizone-induced MS group fed with a zinc-deficient diet (Group 4) (P<0.05). Spinal cord MDA levels of the cuprizone-induced and zinc supplemented MS group (Group 5) were significantly lower than the other MS groups (P<0.05), and GSH levels were higher (P<0.05, Table 5 and Fig. 3).

### 6.3. Interleukin-6 and ZnT3 gene expressions in spinal cord tissue

The highest IL-6 gene expressions and the lowest ZnT3 gene expressions in the spinal cord tissue were obtained in the MS group (Group 3) and the MS groups fed with a zinc-deficient diet (Group 4) (P<0.05). Spinal cord IL-6 gene expressions of the MS group (Group 5) that received zinc supplementation were significantly lower (P<0.05) than the other MS groups (Groups 3 and 4), while ZnT3 gene expressions were higher (P<0.05, Table 6 and Fig. 4).

Additionally, IQR values for ZnT3, IL-6, MDA and GSH parameters

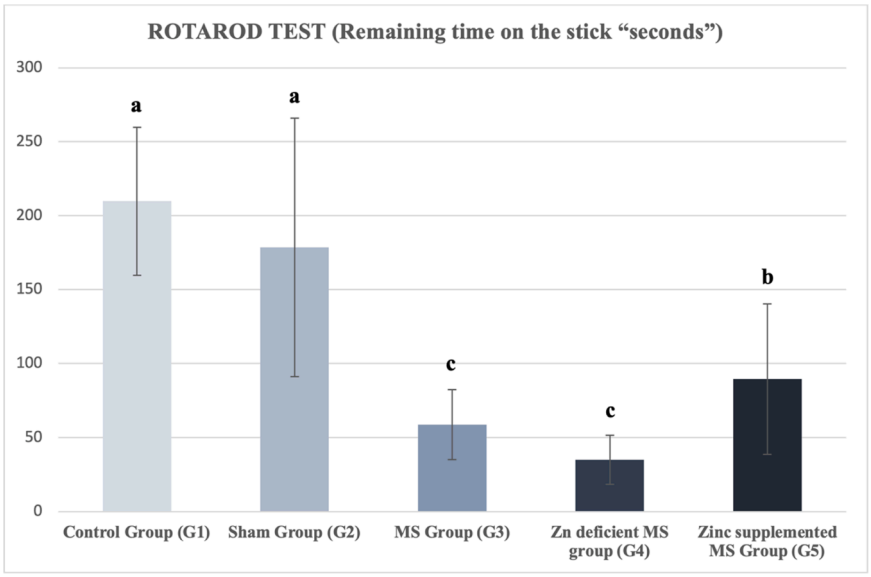


**Fig. 1.** Rotarod test results of the study groups. a>b>c: Means with different letters are statistically significant. (P<0,05).

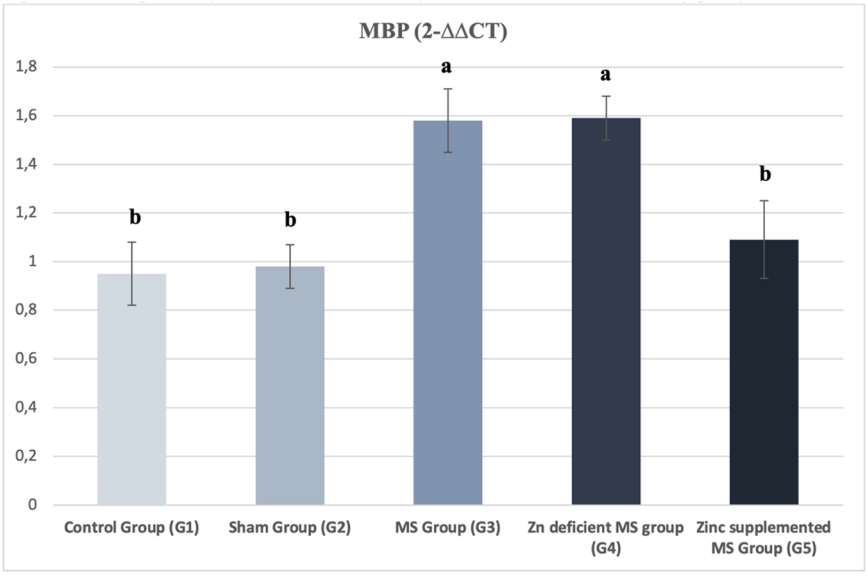


**Fig. 2.** MBP gene expression levels in the study groups. a>b: Means with different letters are statistically significant. (P<0,05).

**Table 3**
Primers used for real-time qPCR.

| Gene | Primer Sequence |
|---|---|
| Znt3 Forward | 5'- ACCACCTTCCTCTTCTCTATCT−3' |
| Znt3 Reverse | 5'-TCCCTTACAGGCTCAAATTCC- 3' |
| İnterlökin−6 Forward | 5'-GGGCTGCGATGGAGTCAGAG−3′ |
| İnterlökin−6 Reverse | 5'-TCCCTCACACAGGGCTCGAC−3′ |
| GAPDH Forward | 5'- GGGCCAAAAGGGTCATCATC −3 |
| GAPDH Reverse | 5'- AACCTGGTCCTCAGTGTAGC - 3 |

**Table 4**
Comparison of Weight Changes of Rats Used in the Study.

| Groups | Rat Weights (grams) Mean±SD |
|---|---|
| Control Group (G1) | 417,28±27,32 **ab** |
| Sham Group (G2) | 427,46±21,50 **a** |
| MS Group (G3) | 406,78±14,84 **ab** |
| Zn deficient MS group (G4) | 373,31±13,92 **c** |
| Zinc supplemented MS Group (G5) | 400,61±16,40 **b** |

a>b>c: The difference between averages with different letters in the same column is important (P<0,05).
**P Values:** G1-G2=0303 G1-G3=0288, G1-G4=0000, G1-G5=0095, G2-G3=0051, G2-G4=0000, G2-G5=0009, G3-G4=0002 G3-G5=0530, G4-G5=0008

are presented in Table 7.

## 7. Discussion

### 7.1. Discussion of body weight changes

The role of zinc in regulating nutrition is critical. Marginal zinc

**Table 5**
MDA and GSH levels in the spinal cord tissue of the study groups.

| Groups | MDA (nmol/gr tissue) Mean±SD | GSH(mg/gr doku) Mean±SD |
|---|---|---|
| Control Group (G1) | 0,14±0,02[b] | 0,28±0,06[a] |
| Sham Group (G2) | 0,15±0,02[b] | 0,31±0,07[a] |
| MS Group (G3) | 0,33±0,03[a] | 0,18±0,07[b] |
| Zn deficient MS group (G4) | 0,31±0,03[a] | 0,18±0,04[b] |
| Zinc supplemented MS Group (G5) | 0,12±0,01[b] | 0,35±0,02[a] |

a>b: The difference between averages with different letters in the same column is important (P<0,05).
**P-Values-MDA**; G1-G2=0966; G1-G3=0000; G1-G4=0000; G1-G5=0756; G2-G3=0000, G2-G4=0000, G2-G5=0230, G3-G4=0833, G3-G5=0000, G4-G5=0000
**P-Values-GSH**; G1-G2=0939, G1-G3=0012, G1-G4=0014, G1-G5=0263, G2-G3=0000, G2-G4=0000, G2-G5=0581, G3-G4=1000 G3-G5=0000, G4-G5=0000

deficiency is associated with decreased appetite as well as low body mass. The most important event in the reduction of body weight in zinc deficiency is changes in the concentration of neurotransmitters in the hypothalamus that regulate food intake and appetite [24]. In this respect, the decrease in body weight of the MS group (G4) fed a zinc-deficient diet in our study can be considered an expected result.

### *7.2. Discussion of MDA and GSH parameters in spinal cord tissue*

MS is a chronic autoimmune disease caused by persistent inflammation in the central nervous system [25]. The most important reason why current treatment strategies in MS are inadequate is that the physiopathological processes underlying the progressive steps in this disease remain unclear [26]. Clinically, one of the common features of the early stages of MS is the imbalance in the pro-oxidant and antioxidant processes in the nervous tissue. This imbalance results in high production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [25–27]. Pathological processes such as demyelination and axonal damage, which are the two most important factors in the pathogenesis of MS, are driven by oxidative stress [28]. For this reason, there is increasing interest in studies investigating the potential therapeutic benefits of strategies that can prevent the increase in different lipid peroxidation products during neuroinflammation in MS [25,27].

In our study, we determined MDA and GSH parameters to determine how dietary zinc status affects lipid peroxidation in spinal cord tissue in a cuprizone-induced rat MS model.

Accordingly, the highest MDA and lowest GSH levels in the spinal cord tissue were obtained in the cuprizone-induced MS group (Group 3) and the cuprizone-induced MS group fed with a zinc-deficient diet (Group 4). Spinal cord MDA and GSH levels of the MS group (Group 5), which was induced with cuprizone and given daily 5 mg/kg ip zinc supplementation in addition to the standard rat chow, were not different from the control groups. This data we obtained shows that zinc supplementation prevents lipid peroxidation by increasing antioxidant activity in the spinal cord tissue in the cuprizone-induced rat MS model.

The suppressed antioxidant activity and increased lipid peroxidation that we obtained in the MS groups that did not receive zinc supplementation (Groups 3 and 4) are compatible with the above-mentioned reports reporting increased tissue damage in MS.

Zinc is an element that has various roles in the pathogenesis of development, injury and neurodegenerative processes [6,7,29]. There is increasing evidence that disorders in zinc regulation occur in patients with multiple sclerosis (MS) [8]. The idea that zinc plays an important role in the pathogenesis of MS arises from its high concentration in the central nervous system and its participation in brain physiology [8]. Zinc involves several major groups of myelin proteins, including myelin

**Table 6**
Interleukin-6 and ZnT3 gene expression levels in the Spinal Cord Tissue of the Study Groups.

| Groups | **Il-6 ($2^{-\Delta\Delta CT}$)** Mean±SD | **Znt3 ($2^{-\Delta\Delta CT}$)** Mean±SD |
|---|---|---|
| Control Group (G 1) | 0,91±0,29**b** | 1,25±0,74**a** |
| Sham Group (G 2) | 0,97±0,30**b** | 1,21±0,64**a** |
| MS Group (G 3) | 1,61±0,50**a** | 0,81±0,56**b** |
| Zn deficient MS group (G 4) | 1,67±0,15**a** | 0,51±0,33**b** |
| Zinc supplemented MS Group (G 5) | 0,97±0,22**b** | 1,41±0,63**a** |

a>b: The difference between averages with different letters in the same column is important (P<0,05).
**P-Values-ZNT3**; G1-G2=1000, G1-G3=0795, G1-G4=0018, G1-G5=0990, G2-G3=0769, G2-G4=0047, G2-G5=0962, G3-G4=0755, G3-G5=0366, G4-G5=0032
**P-Values-IL-6**; 1–2=0996 1–3=0001, 1–4=0000, 1–5=0996, 2–3=0001, 2–4=0000, 2–5=1000 3–4=0989, 3–5=0001, 4–5=0000

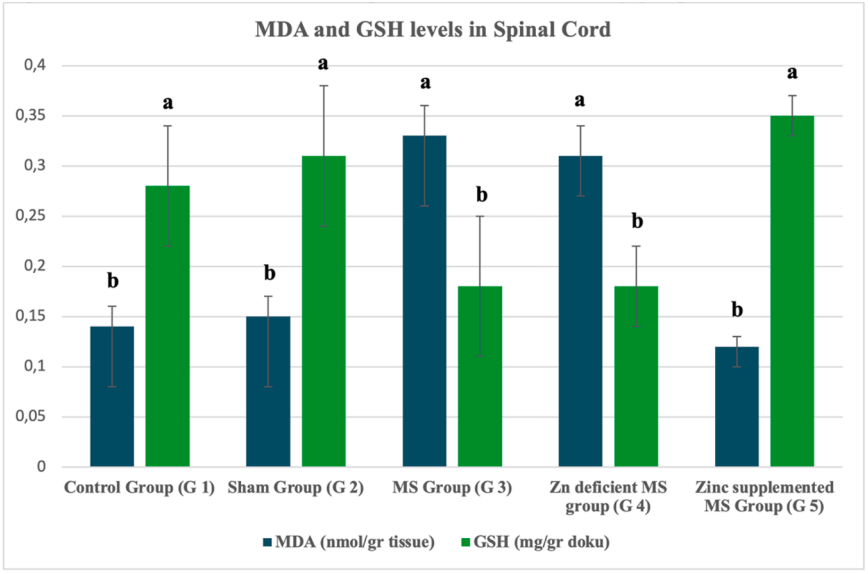


**Fig. 3.** MDA and GSH levels in the study groups. MDA and GSH levels in the spinal cord tissue of the study groups. The MDA values of group 3 and 4 are higher than in the other groups. The GSH values of group 3 and 4 are lower than in the other groups. a>b: Means with different letters are statistically significant. (P<0,05).

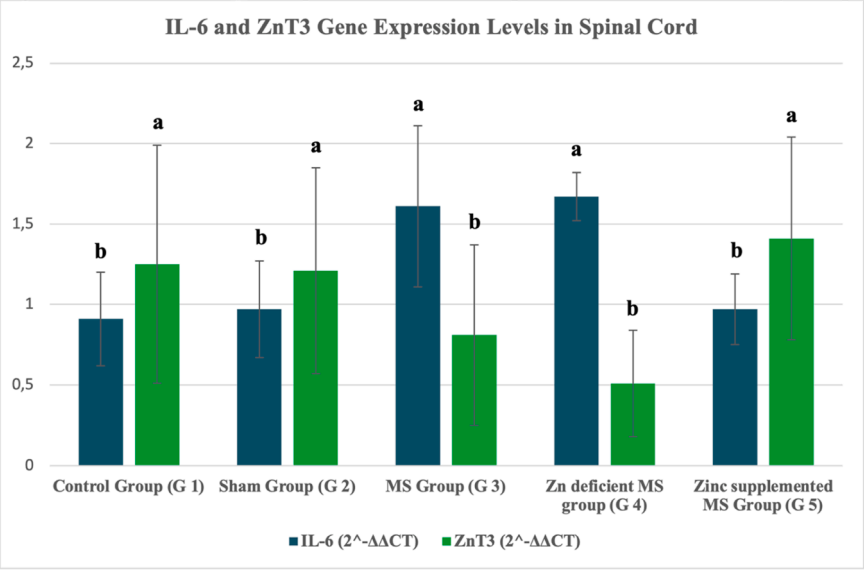


**Fig. 4.** IL-6 and ZnT3 levels in the study groups. Interleukin-6 and ZnT3 gene expression levels in the Spinal Cord Tissue of the Study Groups. The IL-6 values of group 3 and 4 are higher than in the other groups. The ZnT3 values of group 3 and 4 are lower than in the other groups. a>b: Means with different letters are statistically significant. (P<0,05).

**Table 7**
IQR values.

| Descriptive Statistics | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | N | Mean | Std. Deviation | Minimum | Maximum | Percentiles | | |
| | | | | | | 25th(Q1) | 50th (Median) | 75th(Q3) | IQR(Q3-Q1) |
| ZNT3 | 46 | 1,0380 | ,71410 | 0,04 | 2,94 | 0,4001 | 0,9501 | 1,4562 | 1,0561 |
| IL−6 | 46 | 1,2610 | 0,45891 | 0,45 | 2,87 | 0,9986 | 1,1332 | 1,5653 | 0,5667 |
| MDA | 46 | 0,2220 | 0,09514 | 0,11 | 0,38 | 0,1306 | 0,1773 | 0,3269 | 0,1963 |
| GSH | 46 | 0,2622 | 0,09148 | 0,09 | 0,39 | 0,1962 | 0,2683 | 0,3451 | 0,1489 |
| Grup | 46 | 3,17 | 1355 | 1 | 5 | 2,00 | 3,00 | 4,00 | |

basic protein (MBP), which has important functional roles in myelin homeostasis, immune system regulation, and neuronal and oligodendrocyte death. Therefore, it is inevitable that zinc plays critical roles in the pathogenesis of MS [9]. Diseases such as acute brain injury and neurodegenerative diseases are accompanied by impaired zinc regulation [10]. Synchrotron X-ray fluorescence imaging and total zinc content in autopsy samples collected from MS patients show that the content decreases in parallel with myelin loss in the white matter lesion [30]. It is generally accepted that circulating zinc is low in MS patients [9]. It has been shown that serum zinc decreases in autoimmune diseases related to MS [31].

In the current study, we found that ip zinc administration at a dose of 5 mg/kg daily for 8 weeks reversed the deterioration in oxidant-antioxidant parameters in the cuprizone-induced MS group (Group 5). In other words, in the MS group that received zinc supplementation (Group 5), we obtained increased GSH and suppressed MDA levels in the spinal cord tissue when compared to other MS groups (Groups 3 and 4). The antioxidant effect of zinc is well known [16,32–34]. It has been shown that zinc can correct memory disorders by improving antioxidant capacity in brain tissue [35]. Considering that oxidative stress and inflammatory processes are among the critical factors in the secondary injury phase after traumatic spinal cord injury, it is pointed out that zinc may play a very critical role at this stage as a part of the antioxidant defense system [36]. Li et al. [37] reported that zinc contributed to functional recovery by increasing antioxidant activity after spinal cord injury demonstrated. As a result, it can be said that zinc, an antioxidant element, plays a critical role in spinal cord injuries.

The current study reveals that increased oxidative stress in the cuprizone-induced rat MS model is prevented by zinc supplementation. Based on this point, we suggest that zinc supplementation can be used as a potential therapeutic element in preventing increased lipid peroxidation products in the spinal cord tissue in MS.

### *7.3. Discussing IL-6 gene expression levels in spinal cord tissue*

IL-6 is a multifunctional cytokine produced by immune cells and has an important role in the regulation of the immune system [38]. It is indispensable in the development of Th17 cells, antigen-specific cytotoxic T cells and monocytes [38]. In MS patients, the presence and dominant position of IL-6 in acute and chronic active plaques has been demonstrated by immunohistochemistry methods [39]. IL-6 levels in serum and cerebrospinal fluids are significantly higher in MS patients compared to non-inflammatory neurological controls [40]. Similarly, Azzolini et al. [41] reported that IL-6 levels increased in the cerebrospinal fluid of MS patients. Interleukin-6 (IL-6) is an important mediator in the acute phase of inflammatory diseases such as multiple sclerosis [42]. It is suggested that monitoring IL-6 levels, in particular, can be used as a prognostic biomarker in the early stages of multiple sclerosis [39]. Increased IL-6 levels in the cerebrospinal fluid reflect the clinical expression of brain damage, therefore it is noted that IL-6 blockade in MS disease may prevent the development of the disease [43]. As a result, it can be said that there is an increasing interest in researching strategies that suppress IL-6 activation in MS disease [44].

In our study, consistent with the reports given above, we obtained the highest IL-6 gene expression levels in the spinal cord tissue in Groups 3 and 4, which are MS groups. The increased IL-6 levels we obtained in

the spinal cord tissue are also important as they show that the rat experimental MS model occurs with 8-week cuprizone administration.

It has been reported that the application of metallothionein-2, a zinc carrier protein, in an experimental MS model can reduce the severity of MS by reducing IL-6 levels [45]. There are almost no studies investigating the effect of zinc supplementation on the experimental MS model. A study conducted on the basis that therapeutic zinc supplementation could normalize immune functions impaired due to zinc deficiency showed that zinc aspartate treatment could be beneficial in an experimental MS model [46]. It has been pointed out that zinc supplementation may be a promising tool in the treatment of autoimmune diseases such as MS due to its regulatory effect on the immune system [47,48]. Interestingly, based on the beneficial effects of zinc on depression, it has been suggested that zinc supplementation is an appropriate choice for the treatment of depression in MS patients [49].

In the current study, zinc supplementation in the cuprizone-induced MS group (Group 5) significantly suppressed spinal cord IL-6 gene expression levels compared to other MS groups (Groups 3 and 4), reaching control values. The suppressed spinal cord IL-6 levels we obtained with zinc supplementation is a very important finding. As a result, it can be said that zinc supplementation, in addition to the treatment carried out in MS treatment, may be useful in at least reducing the severity of the disease.

### *7.4. Discussing ZnT3 gene expression levels in spinal cord tissue*

Upon synaptic activation, synaptic vesicular zinc is released from presynaptic terminals and then moves to postsynaptic neurons, so zinc can modulate synaptic transmission or increases in pathological levels can lead to neuronal death signals [50,51]. Ionic zinc is localized in synaptic vesicles, ZnT3 is known to be present in the membranes of synaptic vesicles that accumulate zinc and facilitate vesicular zinc accumulation in synaptic vesicles [8]. Genetic deletion of ZnT3 indicates synaptic zinc deficiency in the brain [10]. Since ZnT3 is necessary for synaptic vesicular $Zn2+$ transport, it is thought that the key factor playing a role in the pathogenesis of MS is actually ZnT3 due to its relationship with zinc [10]. Therefore, mice lacking the ZnT3 gene are used in experimental MS models to prevent pathological levels of zinc accumulation in the brain. The aim here is to prevent pathological synaptic zinc accumulation.

In our study, we tried to reveal the relationship between dietary zinc status (dietary zinc deficiency and ip zinc supplementation accepted as physiological dose) and parameters indicating spinal cord tissue damage and ZnT3 gene expression in the cuprizone-induced rat MS model. In this respect, we can say that our study is the first to investigate dietary zinc status in a cuprizone-induced rat MS model.

It has been shown that zinc supplementation at physiological doses may be beneficial in the treatment of autoimmune diseases such as MS by regulating the modulation of pro-inflammatory cytokines [52]. Similarly, zinc treatment reduces the severity of the disease by suppressing the activity of pro-inflammatory cells in experimental MS models [47]. On the other hand, abnormal zinc secretion and pathological intracellular zinc accumulation can increase the severity of MS by triggering events such as matrix metallopeptidase 9 (MMP-9) activation, disruption of the blood-brain barrier, and subsequent immune cell infiltration from peripheral systems [53]. The results of the reports presented above indicate that the zinc-MS relationship is dose dependent.

In our study, ZNT3 gene expression in the spinal cord tissue of experimental MS groups (Groups 3 and 4) that did not receive zinc supplementation was significantly suppressed compared to other study groups. Interestingly, the ZnT3 gene expression levels in the spinal cord tissue of the MS group fed with a zinc-deficient diet (Group 4) were not different from the MS group fed with standard rat chow. We can say this about the data we obtained: While ZnT3 gene expression, a zinc carrier protein, was significantly suppressed in the spinal cord tissue in the cuprizone-induced experimental MS model, zinc-deficient diet did not increase the severity of this suppression. However, in our study, in Group 5, another MS group, zinc supplementation reversed the suppressed ZNT3 gene expression in the spinal cord tissue compared to Group 3 and Group 4, reaching the values of the control groups.

The results of this part of our study indicate that suppressed spinal cord ZnT3 gene expression levels in an experimental MS model can be treated with zinc supplementation.

## 8. Conclusion

When the data of our study are evaluated as a whole;

1.1. The findings of the current study show that lipid peroxidation and IL-6 gene expression increase by suppressing antioxidant activity in the spinal cord tissue in the cuprizone-induced MS model, and spinal cord ZnT3 gene expression is significantly suppressed.
1.2. In the current study, zinc supplementation prevented spinal cord tissue damage in an experimental MS model, reversing the increase in IL-6 gene expression. Again, zinc supplementation increased ZnT3 gene expression, which was suppressed in spinal cord tissue, to control values.
1.3. It can be said that zinc supplementation at a dose of 5 mg/kg in addition to the treatment of MS disease may be useful in reducing the severity of the disease.
1.4. This study is the first to investigate experimental MS model, dietary zinc status, spinal cord tissue damage, IL-6 and ZnT3 gene expression levels.

## Compliance with ethical standards

The study protocol was approved by the Experimental Animals Ethics Committee of Selcuk University Experimental Medicine Research and Application Center (2021–18).

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## CRediT authorship contribution statement

**Rasim Mogulkoc:** Writing – review & editing, Writing – original draft. **Abdulkerim Kasim Baltaci:** Writing – review & editing, Writing – original draft. **Aysenur Feyza Bayiroglu:** Methodology, Data curation. **Gozde Acar:** Methodology, Data curation. **Elif Gulbahce-Mutlu:** Methodology, Data curation. **Saltuk Bugra Baltaci:** Methodology.

## Declaration of Competing Interest

The authors declare that they have no potential conficts of interest to disclose.

## Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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None

*Author contributions*

AFB, GA, EGM and SBB made substantial contributions to the

conception or design of the work; or the acquisition, analysis, or interpretation of data; or the creation of new software used in the work; RM and AKB drafted the work or revised it critically for important intellectual content, approved the version to be published.

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