

Zinc Supplementation Attenuates Pro-Inflammatory Cytokine Responses in a Cuprizone-Induced Rat Model of Multiple Sclerosis

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ABSTRACT

Introduction: Multiple Sclerosis (MS) is a chronic autoimmune disorder marked by neuroinflammation, demyelination, and neurodegeneration. Zinc is crucial for immune regulation, but its impact on inflammatory cytokines in MS is not well understood. This study examined how dietary zinc status influences pro-inflammatory cytokine release in spinal cord tissue using a cuprizone-induced rat model of MS.

Methods: The experimental cohort comprised 46 adult male Wistar rats, randomly allocated into five groups: Control (n=6) and four experimental groups "G" (n=10 each). G1 served as the untreated Control. G2, Sham-MS received daily oral gavage of carboxymethyl cellulose (CMC) solution-used as the vehicle for cuprizone-administered at 1% of their daily feed intake for 8 weeks. G3, G4, and G5 underwent MS induction via daily gavage of cuprizone dissolved in CMC at 1% of daily feed intake over the same period. G4 was maintained on a zinc-deficient diet (50 µg/kg zinc), whereas G5 received intraperitoneal zinc sulfate supplementation at 5 mg/kg/day. Disease progression was assessed by Rotarod performance and Myelin Basic Protein gene

expression. Spinal cord levels of TNF-α, IL-1β, and IL-6 were measured by ELISA.

Results: Multiple sclerosis induction significantly increased TNF-α, IL-1β, and IL-6 levels (p <0.05), especially in zinc-deficient rats. Zinc supplementation attenuated these cytokine elevations, normalizing them to control levels (p <0.05). Functional and molecular data confirmed reduced disease severity with zinc repletion.

Conclusion: Our findings reveal that zinc status is a critical modulator of neuroinflammation in an experimental MS model. Inflammatory cytokine levels in the spinal cord were significantly higher in both MS and zinc-deficient MS groups, confirming local inflammation associated with demyelination. Furthermore, zinc supplementation demonstrated a regulatory effect by suppressing the increase in inflammatory cytokines in the experimental MS model. These results may point to the therapeutic potential of zinc in MS management.

Keywords: IL-1β, IL-6, multiple sclerosis, neuroinflammation, TNF-α, zinc supplementation

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INTRODUCTION

Multiple Sclerosis (MS), which affects the central nervous system through a chronic, autoimmune and neurodegenerative process (1), is distinguished from other neurological conditions by not being a disease of old age. This disorder, most commonly observed in the middle age group of 20 and above, is more common in women than in men when examined in terms of gender distribution (2). The basic pathophysiology of the disease involves demyelination and axonal damage resulting from the immune system's response against myelin (1,2). In addition, oxidative stress is another important factor contributing to the disease process (3). The lack of a definitive cure for MS has led current research to focus on strategies to slow or halt the progression of the disease (4,5). It is accepted that genetic, environmental, and immunological components play a combined role in the etiology of the disease (1,2).

Highlights

- In experimental MS, IL-1β, IL-6, and TNF-α levels are elevated in the spinal cord.
- Zinc deficiency similarly increases cytokine levels.
- Zinc supplementation reduces inflammatory cytokines to control levels.
- Zinc plays an immunomodulatory and anti-inflammatory role in MS.
- Zinc is critical in MS pathogenesis.

In MS and similar central nervous system diseases, inflammation is one of the main determinants of neurodegeneration (4). Immune cells activated during the disease process accumulate in lesion areas and release various pro-inflammatory cytokines (6). Among these cytokines, Interleukin-1 β (IL-1 β) is a potent mediator that increases myelin damage in both demyelinating diseases and neurodegenerative processes (6,7). Experimental studies show that IL-1 β increases the severity of experimental autoimmune encephalomyelitis by activating the Jun N-terminal kinase (JNK) pathway (8). In addition, increased IL-1 β production in non-infectious conditions has been associated with chronic inflammatory processes such as rheumatoid arthritis, inflammatory bowel disease, and tumor development (9,10). IL-1 β is also known to stimulate the production of interleukin-6 (IL-6), which has similar biological effects (7).

Microglial cells, as resident immune elements of the central nervous system, play a crucial role in initiating the inflammatory response. When activated, these cells release various cytokines, primarily IL-6 (11). Overproduction of IL-6 has been associated with cancer and chronic inflammatory diseases, and its uncontrolled synthesis may contribute to several pathological conditions, including MS (7,11). Studies examining the relationship between serum cytokine levels and clinical status in MS patients have shown a positive correlation between increased IL-6 levels and disease severity and frailty (12). Experimental models have also shown that IL-6 levels increase as the disease progresses and peak at specific time points (13).

Another important cytokine involved in MS pathogenesis is Tumor Necrosis Factor- α (TNF- α). This pro-inflammatory molecule, secreted by leukocytes infiltrating the central nervous system, can exert a direct cytotoxic effect on oligodendrocytes and increase glial cell activation (14,15). Both in vitro and stem cell-based studies have shown that TNF- α causes toxicity in oligodendrocyte progenitor cells and inhibits their differentiation (14,16,17). Clinical studies have reported that TNF- α levels are significantly higher in MS patients compared to healthy individuals, and this increase is associated with disease activity (18,19).

Zinc is an essential trace element in the central nervous system, found in both protein-bound and free ionic forms, and involved in the regulation of synaptic transmission and receptor functions (20,21). However, disruptions in zinc homeostasis can contribute to neurodegenerative processes (22). In particular, zinc deficiency can lead to decreased neurogenesis and increased neuronal apoptosis, increasing susceptibility to cognitive impairments and diseases such as MS (21,22). On the other hand, the anti-inflammatory effects of zinc are well-defined; it can reduce neuroinflammation by suppressing the production of cytokines such as IL-1 β , IL-6, and TNF- α (21, 23). In zinc deficiency, increased cytokine release and impaired immune response can further exacerbate the disease process (20–22).

In an experimental autoimmune encephalomyelitis (EAE) mouse model, it has been reported that intravenous immunoglobulins alone are ineffective in reducing disease severity, but when administered in combination with zinc aspartate, they can provide a significant ameliorating effect (23). Similarly, it has been reported that zinc aspartate modulates the immune response by suppressing T cell proliferation and cytokine production in a dose-dependent manner and can reduce disease severity in the EAE model when administered in appropriate doses (24). Kitabayashi et al. (25) showed that zinc modulates T (h)17-mediated autoimmune responses by inhibiting T (h)17 cell development by suppressing STAT3 activation, and that this mechanism may be an important criterion in suppressing autoimmune diseases. It has been shown that disruption of zinc regulation contributes to MS pathogenesis by increasing immune cell infiltration and demyelination via MMP 9

activation and blood-brain barrier damage, while inhibition of vesicular zinc release reduces inflammation, myelin loss and clinical severity in the EAE model (26).

Multiple sclerosis (MS), a chronic and autoimmune disorder affecting the central nervous system, is characterized by processes of neuroinflammation, demyelination, and neurodegeneration (27). There are a limited number of studies investigating the relationship between zinc, which plays a critical role in cellular immune reactions and autoimmune events, and inflammatory cytokines that emerge in MS. The aim of this study is to investigate the effect of dietary zinc deficiency or zinc supplementation on inflammatory cytokine release in spinal cord tissue in a Cuprizone-induced rat Multiple Sclerosis model.

METHODS

Ethical Approval

All experimental phases of this project were carried out at the Selçuk University Experimental Medicine Application and Research Center (SUDAM). The study was approved by the Animal Experiments Ethics Committee of the same center (Approval No: 2021–18).

Experimental Animals and Housing Conditions

Forty-six adult male Wistar rats used in the research were obtained from SUDAM. During the experiment, the rats were kept in controlled environmental conditions at a temperature of 22–25°C, with a 12-hour light/12-hour dark cycle. A continuous negative pressure ventilation system was used in the animal rooms. To minimize the impact on the circadian rhythm, all experimental applications were carried out between 09:00 and 13:00. The animals were grouped into cages of five. The control group consisted of 6 rats, while each of the other experimental groups consisted of 10 animals.

All animal experiments conducted in this study were approved by the Animal Experimentation Ethics Committee of the relevant institution, and the care, experimental procedures, and sacrifice processes of the animals were carried out in accordance with national legislation and international guidelines. All necessary measures were taken to maximize animal welfare and minimize suffering throughout the experiment.

Nutritional Protocol

Two different diets were applied in the study:

1. Standard rat feed provided by SUDAM containing 95.18 mg/kg zinc,
2. Zinc-restricted diet prepared by Arden Research and Experiment (Ankara, Türkiye) containing 50 μ g/kg zinc.

Experimental Design

The experimental animals were randomly divided into five groups:

Group-1, Control: Rats in this group were fed only a standard diet throughout the study period (8 weeks) and were not exposed to any pharmacological or experimental treatments.

Group-2, Sham: Rats were administered a carrier solution containing 1% carboxymethylcellulose (CMC) prepared in distilled water. The administration was performed orally via gavage for 8 weeks, in a volume corresponding to 1% of daily feed intake. The animals in this group were fed a standard diet.

Group-3, MS (Cuprizone): In this group, cuprizone (Bis (cyclohexanone) oxalidihydrazone; Sigma-Aldrich, Supelco-14690) was used to create an

experimental MS model. Cuprizone was prepared in a solution containing 1% CMC and administered orally via gavage for 8 weeks at a rate of 1% of daily feed intake. The animals were fed a standard diet.

Group-4, MS + Zinc Deficiency Diet: Rats in this group were administered cuprizone according to the protocol described in Group 3 and simultaneously given a zinc-restricted diet for 8 weeks.

Group-5, MS + Zinc Supplementation: Rats were administered cuprizone as described in Group 3. In addition, zinc sulfate was administered intraperitoneally at a dose of 5 mg/kg/day throughout the experimental period.

Multiple sclerosis development in animals was determined by Rotarod tests and Myelin Basic Protein (MBP) gene expression analysis (detailed in our published article, which is part of this study) (28).

Spinal Cord Tissue Collection and Preservation

Following the completion of an eight-week experimental procedure, subjects were sacrificed 24 hours after the last application. Surgical anesthesia depth was established with a combination of ketamine (75 mg/kg) and xylazine (10 mg/kg) administered intraperitoneally (i.p.). After euthanasia, spinal cord tissues were rapidly excised and preserved at -80°C until molecular/biochemical analyses were performed.

Pro-inflammatory Cytokine (TNF- α , IL-1 β , and IL-6) Analysis in Spinal Cord Tissue

Prior to analysis, spinal cord samples were washed with cold phosphate-buffered saline (PBS) to remove contaminants and weighed. Tissues were homogenized in cold buffer solution at a 1:9 (w/v) ratio using an ultrasonic homogenizer (Sonic Vibra-Cell).

Tissue TNF- α , IL-1 β , and IL-6 concentrations were determined using commercial ELISA kits, adhering to the manufacturer's protocols (NEPENTHE; Catalog Nos: NE010076403, NE010011903, and NE010013503). Plate washing was performed using a Rayto RT-2600C instrument, and optical density (OD) measurements were determined using a microplate reader (Clariostar, BMG Labtech, Germany). The obtained cytokine data were standardized in pg/mL units.

Statistical Analysis

Data analysis was performed using IBM Statistical Package for Social Sciences (SPSS) program (v. 26.0, IBM Corp., Armonk, NY, USA) software.

The normality of the variables was examined using the Shapiro-Wilk test, and it was determined that the data met the assumptions of parametric tests. Continuous variables are presented as arithmetic mean $\pm$ standard deviation ($\pm$ SD). One-Way Analysis of Variance (ANOVA) was used to determine differences between groups; post-hoc Duncan multiple comparison test was applied to determine the source of significant differences. The statistical significance level was accepted as $p < 0.05$.

RESULTS

Spinal Cord IL-1 β Levels

The highest IL-1 β levels in spinal cord tissue were observed in the MS group (G3) and the MS + Zinc-Deficient Diet group (G4) (403.05 $\pm$ 26.20 pg/mL and 416.22 $\pm$ 28.37 pg/mL, respectively).

In contrast, zinc supplementation (G5) markedly reduced IL-1 β levels (185.45 $\pm$ 18.08 pg/mL), bringing them close to control values observed in G1 and G2 (177.49 $\pm$ 15.35 pg/mL and 180.15 $\pm$ 17.19 pg/mL, respectively).

The differences among the groups were statistically significant ($p < 0.05$) (Fig. 1).

Spinal Cord IL-6 Levels

Spinal cord IL-6 levels were significantly elevated in G3 and G4 (274.64 $\pm$ 25.36 pg/mL and 273.10 $\pm$ 26.40 pg/mL, respectively).

Zinc supplementation in G5 reduced IL-6 levels to 179.17 $\pm$ 18.35 pg/mL, which were comparable to those of the control groups (G1:169.42 $\pm$ 22.08 pg/mL; G2:171.31 $\pm$ 19.62 pg/mL).

These differences were statistically significant ($p < 0.05$) (Fig. 2).

Spinal Cord TNF- α Levels

The highest TNF- α concentrations in spinal cord tissue were detected in G3 and G4 (393.02 $\pm$ 28.52 pg/mL and 404.48 $\pm$ 29.35 pg/mL, respectively).

In the zinc-supplemented group (G5), TNF- α levels (260.95 $\pm$ 21.45 pg/mL) decreased toward control levels (G1:249.52 $\pm$ 24.30 pg/mL; G2:255.44 $\pm$ 23.65 pg/mL).

Group differences were statistically significant ($p < 0.05$) (Fig. 3).

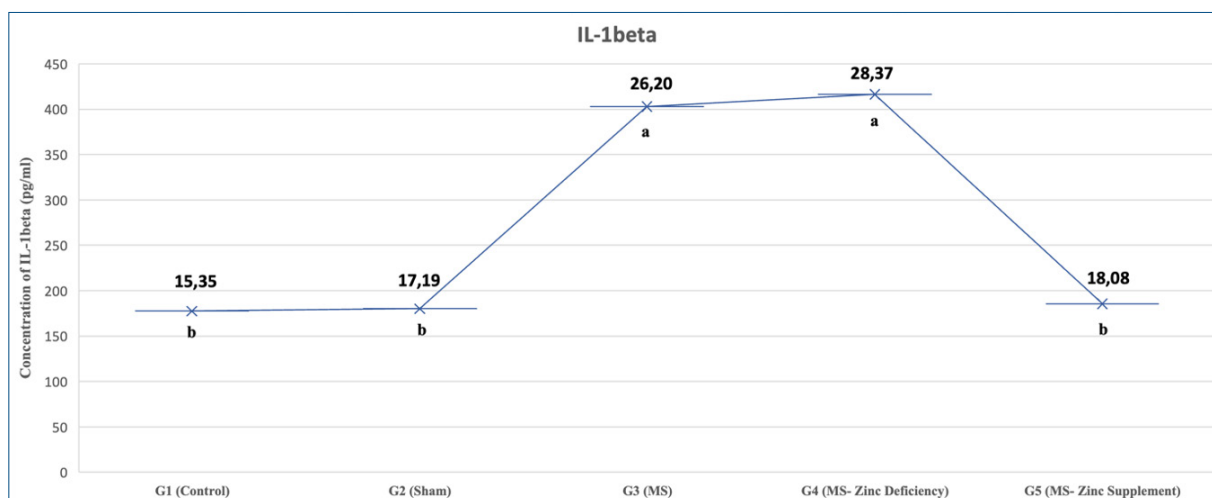


Figure 1. Spinal IL-1 β levels of study groups (pg/ml) (Differences between means indicated by different letters are statistically significant [$P < 0.05$], [a > b]).

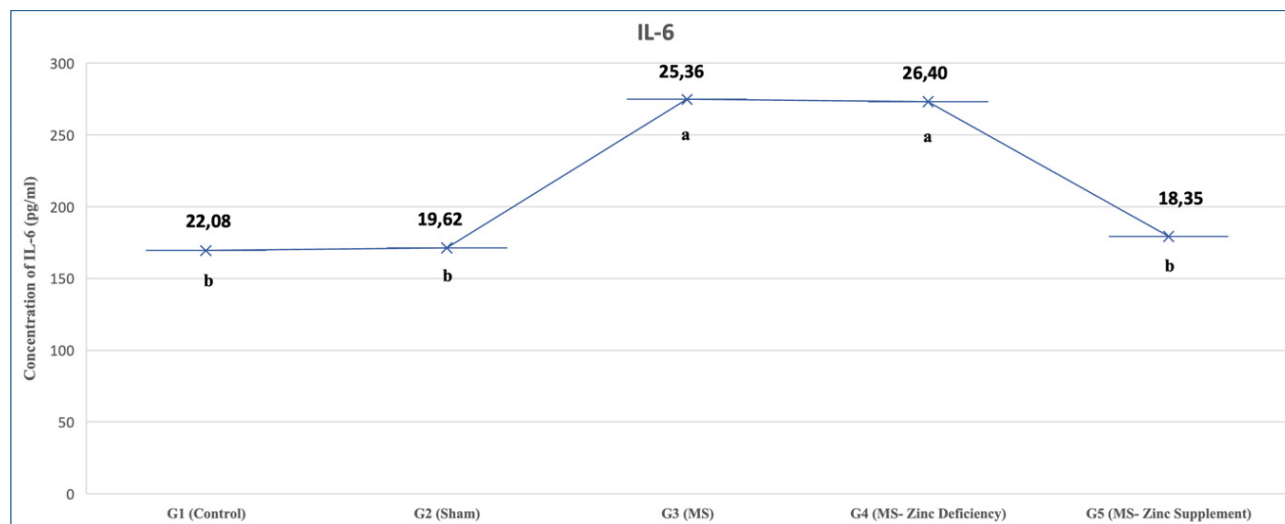


Figure 2. Spinal IL-6 levels of study groups (pg/ml) (Differences between means indicated by different letters are statistically significant [$P < 0.05$], [$a > b$]).

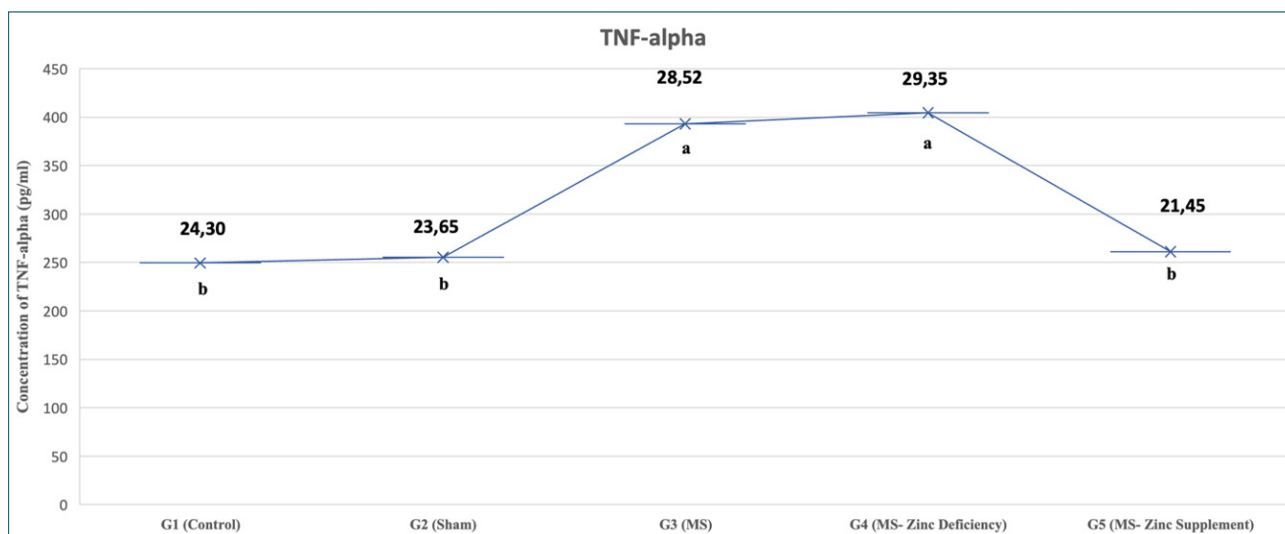


Figure 3. Spinal TNF-α levels of study groups (pg/ml) (Differences between means indicated by different letters are statistically significant [$P < 0.05$], [$a > b$]).

DISCUSSION

Discussion of IL-1 β Levels in Spinal Cord Tissue

The spinal cord is a frequently affected area in MS pathology, and lesions significantly contribute to disability (29–31). In MS, infiltration of immune cells into the CNS leads to myelin and axon damage, particularly in the spinal cord, causing motor, sensory, and autonomic dysfunctions (31). IL-1 β , a pro-inflammatory cytokine, is produced by macrophages, microglia, and monocytes and is found at increased levels in the blood, CSF, and CNS lesions of MS patients; this increase is associated with disease severity and progression (32–34). IL-1 β increases blood-brain and blood-spinal barrier permeability, facilitating leukocyte infiltration and exacerbating neuroinflammation (34,35). It also contributes to demyelination and neuronal damage by triggering glial activation, chemokine production, and inflammatory cascades (32,34). In the EAE model, zinc aspartate has been shown to reduce disease severity, particularly through suppressive effects on the immune response (23–26). In our study, the highest IL 1 β levels in the spinal cord were detected in the G3 and G4 MS groups fed with zinc deficiency, and this finding is consistent with the literature.

Discussion of IL-6 Levels in Spinal Cord Tissue

IL-6 is an important pro-inflammatory cytokine in MS pathogenesis, and its increased levels are associated with disease activity, inflammation, and demyelination (36–38). This study showed significantly increased IL-6 levels in spinal cord tissue in MS model groups (G3:MS-standard diet and G4:MS + zinc deficiency). In contrast, IL-6 levels in the G5 group, which received zinc supplementation, were found to be similar to the control groups (G1–G2). These results suggest that increased IL-6 may be associated with local inflammatory response and oligodendrocyte damage. Zinc deficiency disrupts immune homeostasis by increasing pro-inflammatory cytokine production; in particular, the increase in IL-6 levels has been reported to be associated with the activation of STAT3-mediated signaling pathways (25,39–41). This mechanism explains the high IL-6 levels observed in the G4 group. On the other hand, the inflammatory suppressive effects of zinc supplementation are consistent with the decrease in IL-6 levels. Zinc supplementation has been shown in the literature to reduce IL-6 gene expression and modulate the inflammatory response in EAE models (42–44). Furthermore, zinc is reported to support immune balance by suppressing the Th17 response and increasing regulatory T cells.

In conclusion, our findings demonstrate that zinc status is a significant regulator of inflammation in the MS model and that zinc supplementation may exert an anti-inflammatory effect via IL-6.

Discussion of TNF- α Levels in Spinal Cord Tissue

In this study, spinal cord TNF- α levels were significantly increased in experimental MS groups (standard diet and zinc deficiency) compared to the control group; It has been shown that zinc supplementation suppresses this increase and brings levels closer to control. These findings support the regulatory role of zinc in neuroinflammation. TNF- α is a key proinflammatory cytokine associated with immune dysregulation, demyelination, and tissue damage in models of MS and EAE, and its increased levels are associated with disease activity (45).

Zinc plays a critical role in immune homeostasis; Its deficiency is associated with the increase of pro-inflammatory cytokines such as TNF- α , IL 1 β and IL 6 and impaired immune regulation (39). This explains the high TNF- α levels observed in the zinc deficiency group in our study. In contrast, the normalizing effect of zinc supplementation on TNF α is consistent with previous studies. It has been shown that zinc application suppresses T cell activation and cytokine production in EAE models, and also reduces the Th17 response and increases regulatory T cells (24, 44).

The fact that disruption of zinc homeostasis has also been reported in MS patients supports the importance of this element in neuroinflammation (43,46). Suppression of TNF- α by zinc may also be significant in terms of reducing blood-brain barrier permeability, microglial activation and oxidative damage (45).

In conclusion, while zinc deficiency increases the inflammatory response, zinc supplementation alleviates neuroinflammation by reducing TNF- α levels. These findings suggest that zinc is a potential modulator of immune regulation in MS.

As a result, this study demonstrates that zinc status is a key modulator of neuroinflammation in an experimental model of multiple sclerosis. Both MS and MS model animals fed a zinc-deficient diet showed significantly elevated levels of pro-inflammatory cytokines IL-1 β , IL-6, and TNF- α in their spinal cords, confirming the presence of intense local inflammatory activity associated with demyelination pathology.

Conversely, zinc supplementation effectively attenuated the elevation of IL-1 β , IL-6 and TNF- α , restoring their levels close to those observed in control groups. These findings highlight the immunomodulatory and anti-inflammatory role of zinc in the central nervous system. The results support the concept that zinc homeostasis is crucial for maintaining immune balance and limiting pathological cytokine production in MS-like neuroinflammation.

Overall, our data suggest that zinc deficiency may represent a risk factor that intensifies inflammatory damage in MS, while zinc supplementation may have therapeutic potential as an adjunct strategy aimed at reducing neuroinflammatory burden. Further mechanistic and clinical studies are warranted to clarify the translational relevance of zinc modulation in multiple sclerosis.

Limitations

There are several limitations to consider when interpreting the findings of this study.

First, the sample size in each group was relatively limited, which may restrict the generalizability of the results. Second, only male Wistar rats were used; therefore, potential sex-dependent differences in zinc

metabolism and neuroinflammatory responses were not evaluated. Third, although the cuprizone model is commonly used to mimic myelin loss, it does not fully replicate the complex autoimmune component of human MS, which may limit its applicability to the disease. Furthermore, the study focused on a limited number of pro-inflammatory cytokines (TNF- α , IL-1 β , and IL-6), and other inflammatory and anti-inflammatory mediators were not evaluated. Importantly, the cellular sources of the measured cytokines were not determined. Therefore, it was not possible to distinguish whether these cytokines originated from specific cell populations such as microglia, infiltrating macrophages, astrocytes, or other immune cells; this limits the precise interpretation of the underlying cellular mechanisms. Finally, the trial period was limited to eight weeks, and the long-term effects of zinc supplementation on disease progression were not evaluated.

Future studies with larger cohorts, detailed cell-specific analyses, and comprehensive molecular approaches are needed to better define the therapeutic potential of zinc in MS.

Ethics Committee Approval: The study protocol was approved by the Experimental Animals Ethics Committee of Selçuk University Experimental Medicine Research and Application Center (2021-18).

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Conflict of Interest: The authors declared that there is no conflict of interest.

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