



The Effect Of Vitamin B12 and Folic Acid on Multiple Sclerosis in Mice

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Received: 02 February 2021, Revised: 09 March 2021, Accepted: 17 April 2021

ABSTRACT

Background: Multiple sclerosis (MS) is an autoimmune disease in which a deficiency of vitamin B12 along with folic acid can contribute to its progression. The aim of this study was to investigate the role of these two vitamins in altering myelin base protein (MBP) in the MS model of rats and also to measure some ionic parameters in serum.

Methods: Brain histology was followed by analysis of the relationship between vitamin B12 treatment or folic acid and MBP as well as changes in total protein concentration (TPC). MBP expression was assessed by SDS-PAGE and serum levels of iron. Copper and zinc were also assessed by Duncan test.

Results: MBP expression in cortical extract was increased by simultaneous treatment with vitamin B12 and folic acid compared with the groups treated individually. Histological examinations showed that the highest level of tissue repair was in the same group. There was a significant difference between cortical TPC in the control and treatment of vitamin B12 and folic acid, but serum levels of Fe, Zn and Cu were not significantly different between the groups.

Conclusion: It can be concluded that the combination of these vitamins increases the expression of MBP protein.

Keywords: Multiple Sclerosis, Vitamin B12, Folic Acid, SDS-PAGE.

1. Introduction

Multiple sclerosis (MS) is an autoimmune disease in which the myelin sheath of central nervous system (CNS) is destroyed by the immune system [1, 2]. Vitamin B12 is water soluble, which is especially involved in the normal functioning of the nervous system [3]. Folate acts as a methyl donor in the production of methyl cobalamin, which participates in the methylation of homocysteine and its conversion to methionine [4]. Folic acid plays an essential role in diseases such as MS [5].

Vitamin B12 and folic acid are two micronutrients that are needed for the proper functioning of the immune system [6, 7]. Vitamin B12 plays an important role in the structure and function of the nervous system, and its deficiency leads to myelin depletion and axonal destruction, and causes irreversible damage due to axonal death. Therefore, B12 deficiency can lead to some clinical and paraclinical features similar to those present in MS patients. Some studies have shown that serum B12 levels as well as macrocytosis in MS patients are

significantly reduced, while others do not confirm this finding [8]. There is a possible link between the onset of the first neurological signs of MS and serum levels of vitamin B12 and folic acid of the patient at the time. These findings indicate that lack of vitamin B12 and folic acid and MS have both similar symptoms. In addition, increased intake of vitamin B12 and folate with immunotherapy treatments has shown promising results in MS patients [9]. A meta-analysis study in 2011 was conducted on MS patients to find a relationship between vitamin B12 and other micronutrients. They found a direct relationship between low vitamin B12 levels and the risk of MS [10]. Most of the recent studies have reported increased levels of plasma homocysteine in MS patients; however, a few case-control studies did not find a significant difference in plasma homocysteine levels between their case and control groups, which could be due to patients' specific diets like Mediterranean diet [11]. If only folate supplementation was applied for patients with cobalamin deficiency, it might improve anemia status, but it could not stop the neurological disorders, and if only cobalamin supplementation was used for patients with folate deficiency, it might not treat their anemia [12].

However, the relationship between MS and B12 deficiency is unclear, but several levels of interaction can be suggested. For example, this association is the result of an overlap of autoimmune disorders or indicates an increase in demand for B12 for myelin repair. [8] If the concentration of B12 decreases in a subset of MS patients, treatment is easy and important regardless of the cause. There have been reports of improvement in MS patients treated with B12 [13].

Some ions interfere with the production of myelin and neurotransmitters as well as synaptic transmission [14]. Fe, Cu and Zn are neurotransmitters that can affect the CNS

and recent studies have found a link between the levels of these ions and MS [15]. The aim of this study was to determine the role of vitamins B12 and B9 in combination and separately in changing MBP in MS mice and also to measure ionic parameters by examining their possible effects on MS symptoms.

2. Material and Method

2.1. Animal study

Twenty-five eight-week-old mice were kept at 22 °C for 12 hours. They were then divided into five groups. The control group was given only water and food. The second group of mice received Cuprizone 0.3% (bicyclohexanone-oxalilidehydrazone; Sigma-Aldrich, USA). They received powdered food containing cuprizone with intraperitoneal injection of alginate solvent in the last two weeks. The third group received 0.3% Cuprizone and vitamin B₁₂ ampoules (50 micrograms/kg body weight/once a week). The fourth group received 0.3% cuprizone and folic acid injection (8 mg/kg diet daily) and the fifth group received bercuprizone. 0.3% was given simultaneous intramuscular injection of folic acid, daily (8 mg/kg diet per day) and vitamin B₁₂ (50 µg/kg/weight/once a week) for three weeks. Ethical observation was made in accordance with the standards of the University Ethics Committee and in accordance with the Helsinki Code of Ethics for all animals in order to minimize harm to animals.

2.2. Histological studies

For histology examination, the brains of mice were cut and stained. Finally, the stained tissues were examined under an optical microscope.

2.3. Extraction of MBP protein

Brain samples were frozen, then the cortex was separated and solubilized at

1:10 w/w in a buffer containing 400 μ L of 0.1 M Ethylene diamine tetraacetate (EDTA), 100 μ L of TrisHCl (PH: 6.8), 2800 μ L of NaCl, 1.4 M, SDS %2 and mercaptoethanol 2%. After that, the samples were sonicated in 40 kHz (SOLTEL-3200 L) for 10 minutes. Next, the samples were shaken at 1400 rpm for 10 minutes at 70 °C and finally, the upper liquid was collected and stored at -25 °C [16].

2.4. Measurement of total protein concentration

Protein concentration was determined as standard according to Bradford method using bovine serum albumin. Then UV spectrophotometer analysis was used to measure protein concentration at 595 nm (2550-Shimadzu).

2.5. Biochemical examination of serum

Peritoneal blood samples from mice were collected in plastic tubes and then centrifuged at 3000 rpm. Some elements of mouse serum including zinc, iron and copper were tested by Mandray BS-200 biochemistry analyzer.

2.6. Polyacrylamide gel electrophoresis or SDS-PAGE

SDS PAGE was performed to detect the expression of MBP protein. Electrophoresis of the sample extracted from the cortical extract in polyacrylamide gel was performed in a vertical chamber using 15% polyacrylamide gel with 0.1% SDS. The gels were stained by Kuamasi Blue G250. Molecular markers were used to identify the band.

2.7. Statistical analysis

All measurements were based on Duncan's tests for the discrimination of significance (defined as $P < 0.05$). Data are

represented as the mean \pm standard error of the mean (SEM). Analyses were carried out using the statistical analysis system software (SPSS19).

3. Results

In histological examination of brain tissue of mice in the control group, nerve bands with astrocytes and oligodendroglia cells were observed and no damage was observed in myelin tissue (Figure 1A). In the second group, coprizone treatment resulted in cell damage, which led to sweeping and collapse of the fibrillar structure. In addition to severe myelin damage and destruction of fibrous structure, Gliosis was also observed (Figure 1B). In the third group, reactive gliosis and fibrillar syndrome were diagnosed as symptoms of cell damage, but due to vitamin B12 intake, there was some tissue repair. Mild pycnosis was also observed in the nucleus, which was the result of apoptosis (Figure 1C). In the fourth group, coprizone treatment was observed with tissue damage caused by folic acid injection as well as the structure of background fibrillation and t-cell pigmentation caused by apoptosis and damage to myelin tissue. In this group, tissue repair was less than the previous groups (Figure 1D). Simultaneous treatment with folic acid and vitamin B12 in the fifth group resulted in gliosis changes in the tissue. Although, compared with the previous three cases, the fibrillar structure and myelin tissue are less damaged and isolated, there is a degree of myelin damage as a result of coprizone treatment. Changes related to apoptosis in this group are rare and tissue repair is evident in the fibrillar structure due to the concomitant administration of these vitamins (Figure 1E).

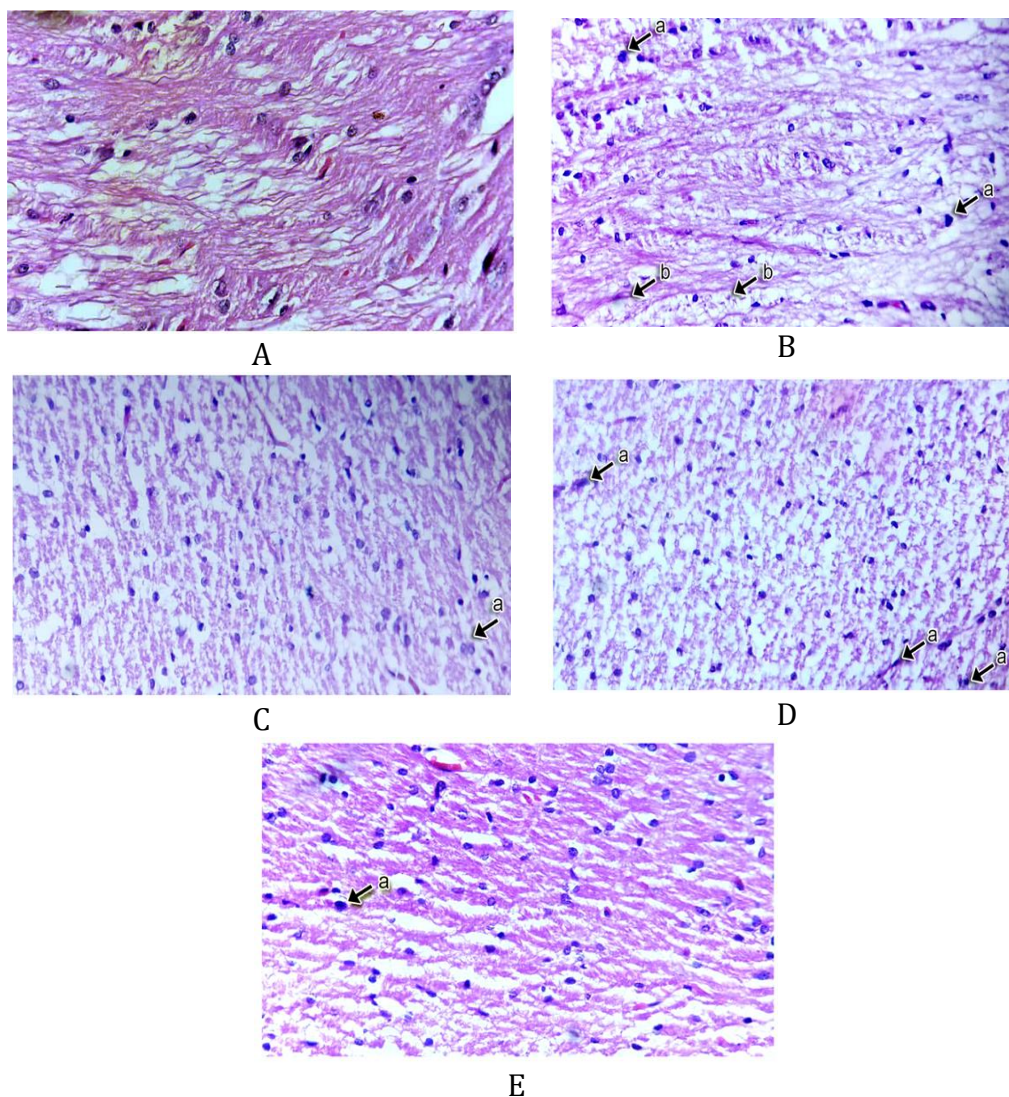


Figure 1. Comparison of Hematoxylin and eosin-stained corpus callosum tissue in different groups. A) Corpus callosum control group (Regular fibrillar structure). B) Corpus callosum Cuprizone group. a. Pycnotic cells. b. vacuolization. (Increase cellularity due to gliosis). C) Corpus callosum Cuprizone+B12 group. a. Pycnotic cells. (Increase cellularity due to gliosis). D) Corpus callosum Cuprizone+B9 group. Pycnotic cells. (Increase cellularity due to gliosis). E) Corpus callosum Cuprizone+B9+B12 group. a. Pycnotic cells. (Increase cellularity due to gliosis) Images are at 400× magnification

3.1. Total protein concentration (TPC)

Although there was a significant difference in brain proteins between the control group and the group consuming cuprizone, no significant difference was observed between the other groups ($P > 0.05$). The control group showed the highest level of protein expression and

the lowest protein expression belonged to the group consuming cuprizone. Among other concentrations of total protein, brain extract in Cuprizone + B12 and Cuprizone + B12 + B9 samples was higher than that of the Cuprizone group ($P < 0.05$), which was significant (Figure 2 & Table 1).

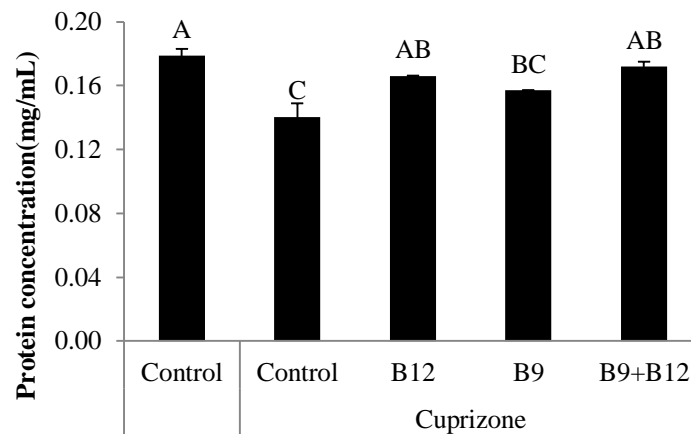


Figure 2. Total protein concentration measurements in control, Cuprizonone, Cuprizonone+B12, Cuprizonone+B9, and Cuprizonone+B9+B12 groups. Data are expressed as an average of three replicates ± standard error of the mean. Non-similar alphabets demonstrate the meaning of the data according to Duncan's (P <0.05) assay.

Table 1. ANOVA table of total protein concentration measurements in control, coprizonone, coprizonone + B12, coprizonone + B9 and coprizonone + B9 + B12.

protein					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.002	4	.001	7.720	.010
Within Groups	.001	7	.000		
Total	.003	11			

3.2. Results of serum biochemical examination

3.2.1. Serum Fe level changes

Serum iron levels were equal in the control group and the group consuming

caprizonone, but were significantly different from other groups (P <0.05). There was no significant difference between the other groups (P>0.05) (Figure 3 & Table 2).

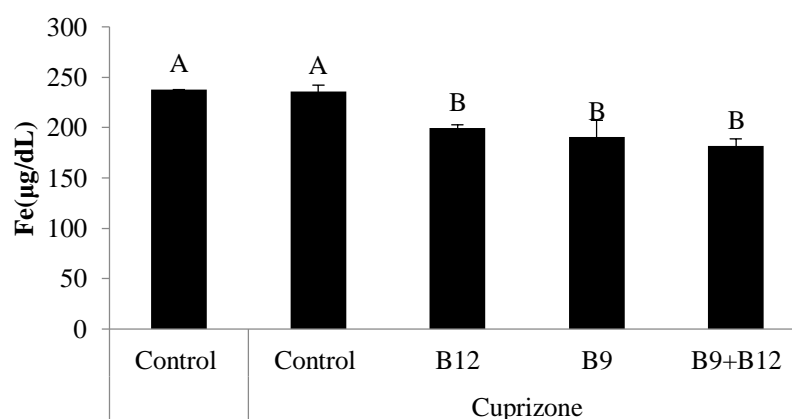


Figure 3. Fe level measurements in control, Cuprizonone, Cuprizonone+B12, Cuprizonone+B9, and Cuprizonone+B9+B12 groups. Data are expressed as an average of three replicates ± standard error of the mean. Non-similar alphabets demonstrate the meaning of the data according to Duncan's (P <0.05) assay

Table 2. ANOVA table of measurement of copper and zinc iron levels in control groups, coprizone, coprizone + B12, coprizone + B9 and coprizone + B9 + B12.

		Sum of Squares	df	Mean Square	F	Sig.
fe	Between Groups	8795.667	4	2198.917	8.626	.002
	Within Groups	2804.083	11	254.917		
	Total	11599.750	15			
cu	Between Groups	8665.364	4	2166.341	1.890	.182
	Within Groups	12606.818	11	1146.074		
	Total	21272.182	15			
zn	Between Groups	2229.271	4	557.318	1.844	.191
	Within Groups	3324.007	11	302.182		
	Total	5553.278	15			

3.2.2. Serum Cu level changes

The group treated with Cuprizone and B9 had the highest serum Cu levels, while the control group showed the lowest Cu

levels and this difference was significant ($P < 0.05$). The difference between the other groups was not significant ($P > 0.05$) (Figure 4).

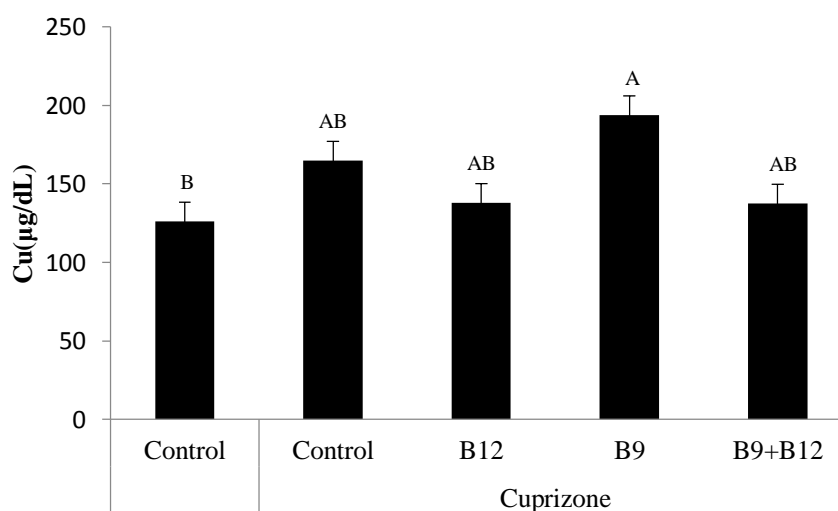


Figure 4. Cu level measurements in control, Cuprizone, Cuprizone+B12, Cuprizone+B9, and Cuprizone+B9+B12 groups. Data are expressed as an average of three replicates ± standard error of the mean. Non-similar alphabets demonstrate the meaning of the data according to Duncan's ($P < 0.05$) assay

3.2.3. Serum Zn level changes

The highest level of zinc was observed in the control group and the lowest level was observed in the groups treated with Cuprizone + B9 and Cuprizone + B12 and

this difference was significant ($P < 0.05$). Zinc levels in the Cuprizone and Cuprizone + B9 + B12 groups were approximately equal ($P > 0.05$) (Figure 5).

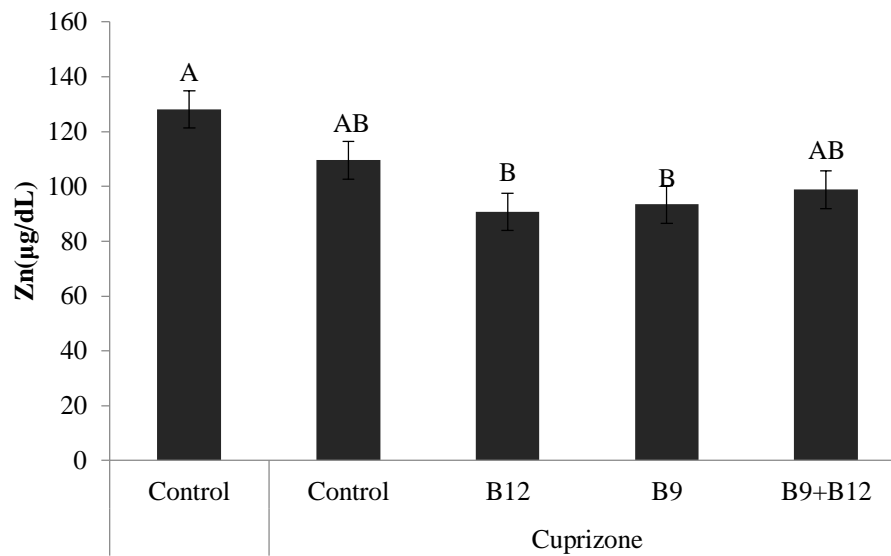


Figure 5. Zn level measurements in control, Cuprizone, Cuprizone+B12, Cuprizone+B9, and Cuprizone+B9+B12 groups. Data are expressed as an average of three replicates \pm standard error of the mean. Non-similar alphabets demonstrate the meaning of the data according to Duncan's ($P < 0.05$) assay.

3.3. Investigating the MBP in the brain cortex using the SDS-PAGE analysis:

In the study of mouse cortex extract using SDS-PAGE, the molecular weight of MBP was between 14-18.5 kDa. The results showed that concomitant use of vitamin B12 and folic acid significantly increased MBP expression compared

with that of the control group. MBP expression levels were significantly reduced in the Cuprizone group of MS patients. MBP expression was approximately equal in the groups consuming vitamin B12 and cuprizone and in the groups consuming vitamin B9 and cuprizone (Figure 6).



Figure 6. SDS-PAGE analysis of mouse brain cortical extracts. Lanes A, B, and C: control group. Lanes D, E, and F: Cuprizone group. Lanes G, H, and I: Cuprizone+B9 group. Lines, J, K, and L: Cuprizone+B12 group. Lanes M, N, and O: Cuprizone+B9+B12 group. Lane P: Ladder or molecular weight markers

4. Discussion

Multiple sclerosis (MS) is known as an autoimmune disease and is one of the most important causes of neurological disability in adults [17]. The potential function of vitamin B in neurophysiology is related to its need for folate and methionine cycles. It is also involved in methylation, production of monoamine

oxidase and storage of lipids, and repair of DNA [18].

Among B vitamins, B12 has attracted the most attention because it is known as a powerful regulator in myelin production [19], some studies have shown the role of vitamin B12 in myelination, regeneration and modulation of MS symptoms [20]. Vitamin B12, as a coenzyme that helps convert homocysteine to methionine, is

involved in the conversion of L-methyl malonone-coenzyme A to succinyl in a folate-dependent reaction [21]. Numerous studies have reported decreased B12 levels in the serum [22, 23] and cerebrospinal fluid [24] of patients with MS. In this study, a combination of vitamin B12 and folic acid reduced the demyelination induced by coprizone and reduced cellulose due to gliosis compared with the groups treated separately.

In a study of MS patients in the attack phase, vitamin B12 levels were lower than those of healthy individuals [25]. In another study, there was no significant difference between serum vitamin B12 in MS and neurological patients with the control group [26]. These results support the hypothesis that patients with vitamin B12 deficiency and people with MS have standard pathological features, but there is no evidence that vitamin B12 is effective in patients with MS [27]. This vitamin is one of the factors in the production of myelin and has regulatory effects on the immune system [28]. The present study showed that vitamin B12 helps to regenerate myelin and it is possible that this vitamin can affect the protein synthesis system [29].

The importance of vitamin B12 and folic acid in the metabolism is due since these vitamins can be used in several biochemical pathways for essential neurological and hematological functions. Several empirical studies have shown that vitamin B12 can protect CNS from high concentrations of glutamate [30]. This suggests that vitamin B12 is not only needed for healthy brain function but also plays a protective role against the toxins that are produced by the brain, although the mechanism of the neurotransmitter's protective effects of the B12 remains obscure [31]. Recent studies have shown that vitamin B12 deficiency can cause an imbalance between growth factors and cytokines,

leading to damage to myelin sheaths. Vitamin B12 deficiency increases the synthesis of factors such as TNF- α , which are toxic to myelin, and can also reduce the production of myelin inhibitory factors such as interleukin-6 and epidermal growth factor [32].

Inconclusive data about the relationship between folate and MS requires more clinical research and a new perspective on the metabolism of folate/folic acid and its importance in neurodegenerative diseases such as MS.

Iron plays a very important role in the normal functioning of the brain. Iron metabolism and abnormal deposition in the brain can be associated with many neurological disorders, including multiple sclerosis (MS) [33]. Serum iron levels in the control group were not significantly different from the coprizone group in the present study, which may indicate an unchanged level of serum iron in MS, although there was a 0.7% increase in serum iron levels in these two groups compared with the other cases. It was shown that serum iron levels in these patients were higher than those of the control group [34] while in another study no change in serum iron levels was reported [35]. The effect of Fe on the development or evolution of MS still needs further investigation, but it is the most likely cause of iron imbalance, which causes inflammation in brain tissue and may eventually lead to the onset of MS [36].

Copper can play a role in MS pathology through excessive accumulation and oxidative damage [37]. Increased copper content occurs through damage to the mitochondrial electron transfer system, active cytochrome oxidase and glia [38]. However, opposite results have also been reported [39, 40]. In this study, a 0.7% increase in serum copper levels was observed in the coprisone-treated group compared with the control group. The highest amount of

copper was in Cuprizone + B9 group with an increase of 0.68% compared with the control group. Cu may play a role in oxidative damage of MS [41]. Also, the increase in copper levels in MS patients compared with the control group may be related to the copper-containing enzyme, cytochrome oxidase [42]. Studies on the effect of zinc supplementation have shown that reduced T cell activation and proliferation could be promising in the future [43]. However, a recent study in patients with MS compared with the placebo group showed no improvement in neurological symptoms after zinc supplementation [44].

The results of this study showed that although the serum zinc level was higher in the control group, it was not statistically significant with an increase of 0.84% compared with the cuprizone and cuprizone + B9 + B12 groups, while considering the treatments of Cuprizone + B9 and Cuprizone + B12 decreased by 0.8% compared with the Cuprizone group, Socha et al found no difference in Zn levels between MS patients and the control group in the study of cerebrospinal fluid [43]. It turns out that the significant effect of Zn in the brain is to help the function of brain enzymes whose fluctuations cause brain dysfunction [45].

SDS-PAGE results showed that the combined consumption of vitamin B12 and folic acid significantly increased MBP, in which the protein band was 14.4 kDa wider than that of the other groups. The protein strips from Cuprizone + Vitamin B12 treatment were clearer than Cuprizone + B9, which could be due to the greater effectiveness of Vitamin B12.

The available data suggest that vitamin B12 and folic acid may have ionic activity in favor of the entry of zinc and copper into cells. In addition, they can act as chelators that kill copper and zinc in plaques and prevent oxidative stress in

the rat brain, thereby reducing peptide oligomerization [45].

5. Conclusion

In this study, we found that the treatment of MS caused by cuprizone with the simultaneous use of vitamin B12 and folic acid could reduce the symptoms of the disease and pathological changes that may be due to their anti-inflammatory, modulatory and antioxidant effects. In the group treated with vitamin B12, the therapeutic effects were better than those of folic acid, but the combination of folic acid and vitamin B12 may play a key role in the expression of MBP and the maintenance of myelin in the brain. Preliminary data show that the effect of MS can be affected by providing enough nutrients (vitamin B12 and folic acid) daily to regenerate myelin. In general, the combination of these vitamins has been shown to increase the expression of MBP protein. Serum levels of iron, zinc and copper were not significantly different in the study groups.

Abbreviation

Not applicable

Conflict of interest

All authors declare no conflict of interest exists.

Consent for publications

All authors read and approved the final manuscript for publication.

Availability of data and material

All data generated during this study are included in this published article.

Ethics approval and consent to participate

No human or animals were used in the present research.

Funding

This work received no financial support.

Ethics declarations.

The authors declare no conflict of interest in financial or any other sphere. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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How to cite this article: Sahar Parchizadeh, Behrooz Salehi-Eskandari. The Effect Of Vitamin B12 and Folic Acid on Multiple Sclerosis in Mice. *International Journal of Advanced Biological and Biomedical Research*, 2021, 9(2), 216-227. Link: <http://www.ijabbr.com/article/243718.html>