Dose-Dependent Role of a New Biotin Compound in Hippocampal Demyelination in Rats

Burak Yulug

Alanya Alaaddin Keykubat Üniversitesi: Alanya Alaaddin Keykubat Üniversitesi

Ertugrul Kilic

Istanbul Medipol University: Istanbul Medipol Üniversitesi

Cemal Orhan

Firat University: Firat Üniversitesi

Besir Er

Firat University: Firat Üniversitesi

Mehmet Tuzcu

Firat University: Firat Üniversitesi

Ibrahim Hanifi Ozercan

Firat University: Firat Üniversitesi

Nurhan Sahin

Firat University: Firat Üniversitesi

Sinan Canpolat

Firat University: Firat Üniversitesi

James Komorowski

Nutrition 21 Inc

Sara Perez Ojalvo

Nutrition 21 Inc

Sarah Sylla

Nutrition 21 Inc

Seyda Cankaya

Alanya Alaaddin Keykubat Üniversitesi: Alanya Alaaddin Keykubat Üniversitesi

Kazim Sahin (✉ nsahinkm@yahoo.com)

Firat University: Firat Üniversitesi <https://orcid.org/0000-0001-9542-5244>

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Abstract

Demyelination is generally observed in different neurodegenerative disorders, including Multiple sclerosis (MS). It is known that high-dose biotin supplementation stabilizes MS progression. To reduce the effective dose of biotin, here we synthesized a new and superior form of biotin, which is a complex of magnesium ionically bound to biotin (MgB) and compared its dose-dependent effect with biotin alone after lyssolecithin (LPC)-induced demyelination in rats. Myelination was studied with luxol fast blue staining and revealed that the most significant remyelination occurred in the MgB groups. We observed dose-dependent improved spatial memory in both biotin and MgB combined animals. Furthermore, we detected decreased inflammatory proteins in both treatment groups, which were more prominent in high-dose MgB treated animals, associated with improved NF- κ B, OP, and MMP-9 proteins. We further analyzed biotin-related proteins and observed that both biotin and particularly MgB reverses the LPC-induced reduction of these proteins. Furthermore, we have observed that biotin but particularly MgB improves neuronal transmission proteins, Synapsin-1, PSD-93, and PSD 95. Moreover, increased BDNF, GAP-43, and ICAM were detected in both treatment groups, especially significant in high-dose MgB treated animals. Increased GFAP, which indicates reactive gliosis, was observed in LPC treated animals, which were reversed particularly by high-dose MgB treatment. The present data reveal the dose-dependent beneficial effect of biotin on demyelination processes. However, a combination of biotin with Mg resulted in a more potent effect than biotin alone. The robust impact of MgB encourages proof-of-concept studies with MgB in human MS patients.

Introduction

Although Multiple Sclerosis (MS) is classically an autoimmune demyelination disorder initiated by migrated T cell-mediated immune responses, a significant body of evidence has indicated that various other CNS cells (*i.e.*, astrocytes and microglia) might also contribute to the progression of this devastating disease [1, 2]. Recent studies have reported that oligodendrocyte damage and axonal degeneration may play an important role in the MS pathophysiology in addition to the demyelination. In line with this, several lines of clinical data have shown that the accumulation of axonal damage is associated with persisting demyelination and progressive neurological dysfunction in MS patients [1, 3–6]. Altogether, these findings concluded that neurodegeneration is a major part of MS pathogenesis, strongly associated with prominent neuroinflammation characterized by the activation of reactive astrocytes and the migration of inflammatory cells via upregulated adhesion molecules, such as intercellular adhesion molecule 1, vascular cell adhesion molecule 1. This interactive process also includes the secretion of proinflammatory cytokines (*e.g.*, TNF- α , IFN- γ , IL-2, IL-12) along with increased matrix metalloproteinases and chemokines, which are responsible for degrading the basement membrane and improving the adherence and extravasation of another inflammatory/immune cells into the central nervous system (CNS) [5, 7].

In addition, immune cells have also been reported to modulate the trafficking of T lymphocytes by activating the nuclear factor- κ B ligand (RANKL), which was ameliorated by the inhibition of nuclear factor kappaB (NF- κ B) ligands in experimental models of MS [8, 9]. Interestingly, osteoprotegerin (OPG) and many other inflammatory proteins, such as Tumor Necrosis Factor α (TNF- α) and Glial Fibrillary Acidic Protein (GFAP), have been shown to modulate the inflammatory response by interacting with NF- κ B ligands in MS [2, 9, 10]. Recently, increasing evidence suggests that Brain-Derived Neurotrophic Factor (BDNF) enhances CNS myelination during development and induces remyelination and synaptogenesis following demyelination in animal models of MS [11, 12]. Moreover, accumulating evidence has shown that BDNF might exert a significant anti-inflammatory effect by interacting with NF- κ B signaling pathways [13–16]. Consistently, the levels of BDNF expression have been reported to be increased in humans with MS, suggesting a compensatory mechanism to promote recovery/repair of damaged neurons [17–19].

Given its recently defined broad brain-protective effects in animal models, its clinically acceptable therapeutic and safety profile, biotin might be a suitable candidate for further evaluation for MS treatment [20]. Many preclinical studies have shown that biotin exerts significant, remyelinating, neuroprotective, and energy-boosting activity [1]. Thus, daily oral biotin

decreased the disability progression of MS in recent clinical trials that enrolled 136 MS patients [21, 22] through a very recent clinical study that showed no effects of high-dose biotin on the disease course in multiple sclerosis patients. It is worth mentioning that most of the MS clinical trials that also used proven disease-modifying drugs do not bring considerable benefits in cognition and clinical outcomes [23]. Taken up, it is still unclear whether these drugs are effective in the cognitive dysfunction of MS patients [24], suggesting the importance of the results of ongoing MS cognition studies [25]. It is difficult to estimate why cognitive dysfunction responds poorly to any pharmacologic intervention. However, cognitive dysfunction associated with different disease conditions, including MS, could be considered a separate pathophysiological entity characterized by specific regional and systemic metabolic dysfunctions. These findings are interesting in the light of recent studies showing that biotin might additionally act as an essential promoter of carboxylases such as pyruvate carboxylase (PC) 3-methylcrotonyl-CoA carboxylase (MCC), propionyl-CoA carboxylase (PCC), and the two isoforms of acetyl-CoA carboxylase (ACC1 and ACC2) which increase aerobic energy production in neurons and oligodendrocytes [1, 3, 26]. Furthermore, there is considerable evidence that improving the bioenergetics in vulnerable regions, such as the hippocampus, might result in significant cognitive improvement [27].

To the best of our knowledge, the combination of magnesium and biotin treatment of the MS model has not been reported before. Beyond this, no study has evaluated the pro-cognitive effects of biotin and magnesium in the MS model, specifically targeting the hippocampus region involved in MS-related cognitive dysfunction. Based on these unique mechanisms of action and previous inconsistent clinical observations [1, 20–22, 28], along with current therapeutic failures in MS-related cognitive dysfunction, this study was designed to assess whether biotin and magnesium could reverse the cognitive disability in rats with strategically LPC-induced hippocampal demyelination. Magnesium Biotinate (MgB), a novel complex of magnesium ionically bound to biotin, is unique in that it significantly increases biotin solubility 40 times greater than that of D-biotin [29]. Therefore, here, we investigated the effects of high and low doses of MgB on the parameters of remyelination, brain energy production, neuroinflammation, and cognitive-neurobehavioral scores.

Material And Methods

Animals and Design

The study was conducted following National Institutes of Health (NIH) guidelines for the care and use of laboratory animals and permitted by local government authorities (Firat University Animal Research Ethics Committee, Approved number: 257169). All rats were housed at 22–24°C on a constant 12-h light (07:00–19:00 hours) and dark (19:00–07:00 hours) cycle. A total of 42 male Wistar albino rats weighing 250–300 g were randomly divided into six groups (n=7 per group).

(i) Vehicle control group, as a LPC solvent, 1.5 µl isotonic saline was injected both hippocampus, and five days later, animals received 1 ml isotonic saline as a solvent of biotin per oral gavage for 4 weeks; (ii) LPC group, 1.5 µl of LPC 1% was injected into the both hippocampus, and five days later, animals received 1 ml isotonic saline as a solvent of biotin per oral gavage for 4 weeks; (iii) Biotin1 (B1) group, 1.5 µl of LPC 1% was injected into both hippocampus, and five days later, animals received 0.9 mg/rat/day biotin1 in a 1 ml isotonic saline per oral gavage for 4 weeks; (iv) Biotin2 (B2) group, 1.5 µl of LPC 1% was injected into both hippocampus, and five days later, animals received 9 mg/rat/day biotin2 in a 1 ml isotonic saline per oral gavage for 4 weeks; (v) MgB1 group, 1.5 µl of LPC 1% was injected into both hippocampus, and five days later, animals received 0.9 mg/rat/day MgB1 in a 1 ml isotonic saline per oral gavage for 4 weeks; (vi) MgB2 group, 1.5 µl of LPC 1% was injected into both hippocampus, and five days later, animals received 9 mg/rat/day MgB1 in a 1 ml isotonic saline per oral gavage for 4 weeks. The amount of biotin and MgB were calculated based on 30 and 300 mg/d needed for a 70-kg adult human after adjusting doses based on metabolic body size [30, 31].

Induction of Demyelination

Demyelination was induced using a stereotaxic injection of lysolecithin (LPC) as earlier defined [32]. For this purpose, animals were anesthetized and placed in a stereotaxic frame in a flat skull position. The animals' skull was drilled, and a single dose of 1.5 μ l LPC 1% (Sigma, St. Louis, MO, USA) in 0.9% saline was injected bilaterally into the around CA1 area of hippocampi, using appropriate stereotaxic coordinates (AP = -3.6 from bregma; ML = $\pm$ 1.6; DV= -3.2 from dura surface) [32]. Control rats were received an equal volume of sterile saline into the CA1 area. The needle was held in place for an additional 3 minutes to equilibrate tissue and inject the solution to prevent the possible reflux through the needle tract.

Analyzes of Spatial Memory and Learning

For this purpose, the Morris water maze test was done as defined earlier [33]. In brief, the water maze test was done in a 160 cm diameter circular black pool containing water (24 $\pm$ 2°C) that had been colored with a non-toxic black dye to contrast the rat. A 12-cm-diameter black-colored round platform was placed 1.5 cm below the water surface. All animals were positioned daily into the water maze room for 1 h before the trial. There has been given a maximum time of the 60s to rats for finding the hidden platform and allowed to remain on the platform for 30s. Animals were guided to land on the platform if they failed to find the platform within the 60s. The rats were given a daily session of four trials for six consecutive days. The swimming pathway and latency in locating the hidden platform were recorded for each trial. On the seventh day, the number of crossings and the percentage of time spent in the target quadrant was tested after removing the platform.

At the end of the study, the blood samples were collected by cardiac puncture after an overnight fast, and all rats were sacrificed by cervical dislocation. The brain samples were removed after sacrificing the animals.

Analyzes of Biochemical Parameters

Serum glucose, total cholesterol, triglyceride, AST, ALT, creatinine, and urea were detected by a biochemical analyzer (Samsung Electronics Co, Suwon, Korea). To detect serum and brain magnesium levels, 0.3 g of the brain and 0.5 ml of serum samples were digested with 5 ml concentrated nitric acid in a Microwave Digestion System (Berghof, Eningen, Germany) for 30 minutes and diluted 1:10 with distilled deionized water. Lanthanum chloride (Merck, Darmstadt, Germany) was added as an interference suppressant for Ca and Mg analyses. The heating program employed as described in the oven's user manual. Mg levels were measured by atomic absorption spectrometry (AAS, Perkin- Elmer, Analyst 800, Norwalk, CT, USA) with flame atomization in an acetylene-air via recognized and fully confirmed procedures at the 285.2 nm wavelength with the Zeeman background correction. The method was verified with certified reference materials (bovine muscle BCR 184), and the accuracy was 95% [34].

For serum biotin levels assay, samples were ultrafiltered as previously defined [35]. Tissue samples (300 mg) were processed in 5 ice-cold homogenization buffers and centrifuged. Before the biotin measurement, the supernatant was ultrafiltered. The pellet was brought to a concentration of 40 g/L in a homogenization buffer. The tissue samples were promptly frozen in a mixture of dry ice and isopropanol and stored at -80°C until analyzing. The biotin level was determined by HPLC (Shimadzu, Kyoto, Japan) as previously described, with minor modifications [36, 37]. The reversed-phase column used was a C18-ODS-3 column, and the biotin-containing chromatography fractions were dried under a stream of nitrogen before the assessment. For malondialdehyde (MDA) analyses, brain tissues were homogenized in ice-cold phosphate buffer solution for 5 min using both an ultrasonic and a mechanic homogenizer and then centrifuged, and the protein content in the supernatant was determined using nanodrop spectrophotometry (MaestroGen, Las Vegas, NV, USA). Then, an HPLC apparatus of Shimadzu UV-vis SPD-10 AVP detector, a CTO-10 AS VP column, and 30 mM KH₂PO₄ and methanol (82.5: 17.5, v/v, pH 3.6) at a flow rate of 1.2 ml/min were used (Shimadzu, Japan). Column waste was monitored at 250 nm.

Histopathological Study

Brain samples were post-fixed in 4% paraformaldehyde at 4°C for 24 h. Haematoxylin and eosin (H&E) staining were used to evaluate the pathological changes. Luxol fast blue staining procedure was used to determine the demyelination based on the earlier studies [32, 38]. After dehydration with different alcohol gradients, the samples were cleared by incubation in xylene, embedded in paraffin, and blocked. Serial coronal sections (6 µm) were taken using a microtome. Sections were stained with hematoxylin and eosin or luxol fast blue using standard protocols. An independent researcher evaluated histopathological changes according to the standard.

Western Blot Analysis

Brain tissue harvested from hippocampi was analyzed by the Western blot method [39]. Briefly, tissue samples of each group were pooled and lysed in a buffer containing enzyme inhibitors.

Total protein content was assessed using the Qubit 2.0 Fluorometer (Invitrogen, CA, USA). Twenty µg samples were electrophoresed and then transferred to a nitrocellulose membrane using the Trans-Blot Turbo Transfer System. After that, membranes were blocked for one h at room temperature, washed, and incubated overnight with AC, ACC1, ACC2, PC, PCC, presynaptic synapsin I, postsynaptic PSD95, PSD93, IL-17, IL-6, TNF- α , GFAP, GAP43, ICAM-1, BDNF and CXCL 16 (Abcam, Cambridge, UK). Then, membranes were washed with TBS-T, incubated with a rabbit secondary (Abcam, Cambridge, UK) antibody for 1 h at room temperature. Individual blots were performed at least three times. Protein loading was controlled by stripping and reprobing the nitrocellulose membrane with an anti- β -actin antibody (Abcam, Cambridge, UK). Protein levels were analyzed densitometrically using an image analysis system (Image J; National Institute of Health, Bethesda, MD, USA), corrected with values determined on β -actin blots, and expressed as relative values compared with the control group.

Statistical Analysis

Statistical analysis was performed using SPSS (version 15, SPSS Inc., Chicago, USA) software. Data were evaluated by one-way ANOVA followed by Tukey *post hoc* test (SAS Institute: SAS User's Guide: Statistics) and expressed as mean $\pm$ standard error mean (SEM). Throughout the study, p values <0.05 were considered statistically significant.

Results

Bodyweight and Biochemical Parameters

We analyzed the possible side effect of LPC, biotin, and MgB treatments. We have observed that LPC reduces body weight compared with vehicle control (Table 1). High dose biotin but particularly both doses of MgB improved weight loss of animals. We have not seen any significant difference in glucose concentration, total cholesterol, triglyceride, AST, ALT, creatinine, and urea levels (Table 1).

Table 1

Effects of biotin and magnesium biotinate (MgB) supplementation on body weight and various biochemical parameters in lysolecithin (LPC)-induced demyelination of rats.

Items	Groups					
	Control	LPC	LPC+B1	LPC+B2	LPC+MgB1	LPC+MgB2
Body weight, g	290.71±5.55 ^a	251.33±7.26 ^d	259.57±2.81 ^{cd}	264.67±1.38 ^{bcd}	272.57±1.99 ^{bc}	281.86±3.02 ^{ab}
Glucose, mg/dL	89.71±3.46	87.73±3.65	87.41±5.08	91.45±3.79	89.43±4.00	86.71±3.40
T-C, mg/dl	76.61±4.83	76.30±4.45	76.16±5.17	74.78±4.20	70.94±4.30	71.50±1.96
Triglyceride, mg/dl	57.71±4.62	58.17±4.81	56.71±3.15	57.83±2.02	57.29±3.89	55.57±1.48
AST, U/L	230.29±8.58	241.67±13.15	238.86±11.16	236.83±8.83	233.86±11.16	232.00±9.87
ALT, U/L	60.29±4.42	66.83±6.82	67.57±7.12	64.83±5.79	62.71±4.13	63.29±4.37
Creatinine, mg/dl	0.25±0.01	0.27±0.02	0.26±0.01	0.26±0.01	0.24±0.01	0.24±0.02
Urea, mg/dl	26.01±5.11	28.85±4.59	28.56±4.26	25.10±3.80	25.86±2.51	24.86±1.64
LPC: lysolecithin; B1: Biotin 0.9 mg/rat/day; B2: Biotin 9 mg/rat/day; MgB1: Magnesium biotinate (Biotin 0.9 mg/rat/day); MgB2: Magnesium biotinate (Biotin 9 mg/rat/day). T-C: Total cholesterol; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase. Data presented as mean and standard error. Different superscripts (a–c) indicate group mean differences (p < 0.05).						

We further analyzed serum and brain Mg levels. LPC treatment reduced Mg levels in both serum and brain tissue (Table 2). Interestingly, both serum and brain levels of biotin were reduced in the LPC treated animals, which were reversed by supplementation of both biotin and MgB (Table 2). We further analyzed malondialdehyde (MDA) levels which were increased by LPC treatment and reversed by biotin- but particularly by MgB treatments (Table 2).

Table 2

Effects of biotin and magnesium biotinate (MgB) supplementation on serum and brain magnesium, biotin and malondialdehyde concentrations in lysolecithin (LPC) induced demyelination of rats.

Items	Groups					
	Control	LPC	LPC±B1	LPC±B2	LPC±MgB1	LPC±MgB2
Serum Mg, mg/dL	2.85±0.07 ^c	2.11±0.03 ^d	2.17±0.04 ^d	2.09±0.04 ^d	3.62±0.04 ^b	6.01±0.04 ^a
Brain Mg, µg/g	150.70±2.44 ^b	115.57±3.96 ^c	119.41±1.71 ^c	118.38±3.58 ^c	161.58±0.57 ^b	187.77±2.33 ^a
Serum Biotin, nmol/L	62.53±2.62 ^c	34.47±1.25 ^d	57.56±1.92 ^c	81.92±2.15 ^b	65.13±1.93 ^c	102.17±2.82 ^a
Brain Biotin, nmol/g	0.182±0.002 ^b	0.104±0.005 ^e	0.151±0.001 ^d	0.187±0.003 ^b	0.168±0.001 ^c	0.199±0.002 ^a
Serum MDA, nmol/mL	1.30±0.04 ^e	3.08±0.11 ^a	2.70±0.05 ^b	2.53±0.07 ^b	2.21±0.04 ^c	1.84±0.04 ^d
Brain MDA, nmol/g	2.92±0.05 ^e	7.37±0.21 ^a	6.25±0.08 ^b	5.84±0.07 ^b	5.10±0.10 ^c	4.55±0.09 ^d
LPC: lysolecithin; B1: Biotin 0.9 mg/rat/day; B2: Biotin 9 mg/rat/day; MgB1: Magnesium biotinate (Biotin 0.9 mg/rat/day); MgB2: Magnesium biotinate (Biotin 9 mg/rat/day). MDA: Malondialdehyde. Data presented as mean and standard error. Different superscripts (a–c) indicate group mean differences (p < 0.05).						

Spatial Memory

Entries to target quadrant and probe trial in the Morris water maze: In evaluating the effects of biotin and MgB supplementation on Entries to target quadrant and probe trial in the Morris water maze task in LPC-induced demyelination of rats, we found that LPC-induced impairment behavioral parameters were dose-dependently and significantly improved after the application of biotin and MgB (Fig. 1A, B; LPC+B1 vs. LPC+B2, p<0.05; LPC+MgB1 vs. LPC+MgB2, p<0.05).

Latency to find the hidden platform in the Morris water maze task: In evaluating the effects of biotin and MgB supplementation on latency to find the hidden platform from the first day to the fifth day in the Morris water maze task in LPC-induced demyelination of rats, we have found that LPC-induced impairment in behavioral parameters was dose-dependently and significantly improved after the application of biotin and MgB (Fig. 1C; LPC+B1 vs. LPC+B2, p<0.05; LPC+MgB1 vs. LPC+MgB2, p<0.05).

Histopathology

First, we have observed that LPC causes diffuse hippocampal degeneration, stained by H&E (Fig. 2A). There was slight edema, infrequent lymphocytic infiltration, and remarkable degeneration of pyramidal cells around the CA1 region of the hippocampus in the LPC group. In the LPC+B1 and LPC+B2 groups, mild edema and mild degeneration of pyramidal cells were detected around the CA1 region of the hippocampus. However, the LPC+MB1 group had only mild edema. In the MgB2 group, a normal histological appearance of the hippocampus was detected.

There was no hippocampal demyelination in saline-treated controls, while LPC-induced remarkable demyelination mainly around CA1 regions in LPC treated animals compared to the control group (Fig. 2B). Biotin or MgB treatment at both doses significantly decreased the demyelination in LPC treated animals. However, significant myelination was detected in the MgB2 group (Fig. 2B).

Inflammatory Markers

In evaluating the effects of biotin and MgB supplementation on inflammatory parameters in the brain, we have found that IL-6, IL-17, TNF- α , chemokine (C-C motif), ligand 3 (CCL-3), chemokine (C-C motif) ligand 5 (CCL-5), and chemokine (C-X-C motif) ligand 16 (CXCL-16) protein levels were dose-dependently and significantly decreased after the application of B1, B2, MgB1, and MgB2 although IL-17 and CCL-5 levels were statistically insignificant between the LPC-B1 and LPC-B2 groups, and LPC+MgB1 and LPC+MgB2 groups, respectively (Fig. 3; $p>0.05$).

Further analysis on NF- κ B, osteoprotegerin (OPG), and matrix metalloproteinase 9 (MMP-9) revealed that NF- κ B levels were dose-dependently and significantly decreased within the groups (Fig. 3; LPC+B1 vs. LPC+B2 and LPC+ MgB1 vs. LPC+MgB2, $p<0.05$) while OPG and MMP-9 levels were not affected by increasing the dosage of biotin (LPC+B1 vs. LPC+B2, $p>0.05$). Additionally, OPG levels responded well to the combination therapy and showed statistically increased levels after increasing the biotin dosage of MgB2 (LPC+MgB1 vs. LPC+MgB2, $p<0.05$)

Biotin-Related Enzymes

In evaluating the effects of biotin and MgB supplementation on brain acetyl CoA carboxylase 1 (ACC-1), acetyl CoA carboxylase 2 (ACC-2), pyruvate carboxylase (PC), propionyl-CoA carboxylase (PCC), 3-methylcrotonyl-CoA carboxylase (MCC), we found that there were no dose-dependent increases in biotin-related enzymes after the application of low (B1) and high doses of biotin (B2) (LPC+B1 vs. LPC +B2, $p>0.05$) except the PCC group which showed no statistically significant increase after increasing the biotin dosage (Fig. 4; LPC+B1 vs. LPC+B2, $p<0.05$). In comparing the effects of MgB1 and MgB2 on biotin-related enzymes, we have revealed that there were significant increases in all biotin-related enzymes (ACC1, ACC2, PC, PCC, MCC, $p<0.05$) except for the PCC (LPC+B1 vs. LPC+B2, $p>0.05$) and MCC enzymes (LPC+B1 vs. LPC+B2, $p>0.05$) in the only-biotin groups (Fig. 4).

BDNF, GAP-43, GFAP, and ICAM Proteins

In evaluating the effects of biotin and MgB supplementation on the BDNF, GAP-43, GFAP, and ICAM, we revealed that growth factor BDNF and GAP-43 levels were dose-dependently increased after biotin (LPC+B1 vs. LPC+B2, $p<0.05$) and MgB supplementation (Fig. 5; LPC+MgB1 vs. LPC+MGB2, $p<0.05$). Conversely, GFAP levels were dose-dependently decreased in biotin (LPC+ B1 v.s LPC+B2, $p<0.05$) and particularly MgB groups (LPC+MgB1 vs. LPC + MGB2, $p<0.05$).

Synaptic Transmission Proteins

In evaluating the effects of biotin and MgB supplementation on brain Synapsin-I (Fig. 6A), postsynaptic density protein 93 (PSD-93, Fig. 6B), and postsynaptic density protein 95 (PSD-95, Fig. 6C) proteins, we have revealed that synapsin was significantly increased in LPC +MgB1 group in compared to the LPC+MgB2 group while the levels of PSD-93 was significantly increased in the LPC+B2 group compared to the LPC+B1 group ($p<0.05$). In contrast, PSD-95 levels were unchanged in the LPC+B2 group compared to the LPC+B1 group ($p>0.05$), while the levels of all synaptic proteins were significantly increased in the LPC+MgB2 group compared to the LPC+MgB1 group ($p<0.05$; Fig. 6).

Discussion

Although many therapeutic options offer some short and long-term immune-modulating effects, these drugs do not exert clinical sustainable neuroprotective effects and thereby fail to improve the long-term disease course in MS [21]. Additionally, no pharmacological agent still exerts beneficial effects in MS-related cognitive impairment. Here, the true challenge with translational MS studies is in connecting the preclinical results to human biological processes, which are complex and can involve combinations of many key pathophysiological components, acting simultaneously and feeding back on one another [40, 41]. Consequently, the complexity of the resulting pathophysiology is such that therapeutic interventions that act on singular pathways may not provide adequate benefit. This led to hypothesize that alternative drugs, including nutritional supplements targeting multiple pathways, may be needed to improve the disease course. Our data show for the first time that magnesium and high-dose biotin was the most effective combination in enhancing

cognitive scores and restoring myelination (OPG I) and inflammatory parameters (ILs, TNF- α , chemokine, NF- κ B, and GFAP) without producing any side effects. Indeed, improved cognitive scores in the magnesium and high-dose biotin group were correlated with more significantly increased bioenergetic mechanisms, including the upregulation of critical biotin-related enzymes, such as ACC-1, ACC-2, PC, PCC, and MCC, which are involved in cognitive processes.

According to the previous effects of BDNF on synaptogenesis, cognition, and learning, we have also analyzed the expression of BDNF, GAP43, ICAM, and synaptic proteins (i.e., PSD 93, PSD 95, and synapsin). In line with hitherto studies, we observed that BDNF levels and markers for axonal regeneration and synaptic remodeling [42–44] were dose-dependently increased after the treatment with biotin and MgBiotin. We next explored whether the pro-cognitive potential of BDNF and other neuro-regeneration markers might still be adequate to alter the neurobehavioral outcomes in the whole study groups. Not surprisingly, we have observed that biotin and magnesium biotinate combination dose-dependently improved cognitive scores, which is in line with the effects of BDNF on learning, memory, and depression [43, 45]. More interestingly, we have noticed that improved neurobehavioral outcomes were also associated with improved remyelination process after biotin and magnesium application. Again, this might suggest interactive processes between re-myelination, synaptogenesis, and (region-specific) energetic requirements involved in improved neurological status. For instance, the different structural expression patterns of the biotin-related enzyme and varying energy requirements of neuronal structures might be responsible for additive beneficial effects. A good example could be acetyl-CoA carboxylase1 and acetyl-CoA carboxylase2, which are expressed in oligodendrocytes and induce myelin repair by supporting the fatty acid synthesis, whereas other biotin-related enzymes are primarily expressed in neurons and protect axons by enhancing energy production [1, 46, 47].

Although there are inconclusive results regarding the routine clinical use of biotin in MS for safety reasons, our results have revealed that serum levels of magnesium and biotin were directly correlated with the brain concentrations of magnesium biotinate. Furthermore, we have observed no significant differences in body weight and biochemical parameters after applying biotin with magnesium. Since poor oral bioavailability can not only result in low efficacy but also lead to some unpredictable side effects, our present data might indicate that the combination of Mg and Biotin might exert stronger bioavailability than other formulations without producing any side effects. From the translational point of view, when our results were compared with data from recent MS clinical trial results, where high-dose biotin did not seem to be associated with a beneficial effect, the main results presented here indicate that low and high doses of magnesium-biotin exerted a significant beneficial impact on myelination, inflammation, regeneration, and neurobehavioral scores. Considering that cognitive impairment is the main factor influencing the quality of life in MS patients [48], our present data is highly relevant for understanding the mechanism of cognitive impairment in these specific groups of patients. From another point of view, biotin-based treatment options, which led to increased availability of alternative energy sources, have been increasingly recommended as a therapeutic tool in degenerative cognitive diseases[49]. Although we have finally noticed that magnesium and high dose biotinate was the most effective combination in reducing the injury markers and functional outcomes, it was interesting to observe that magnesium significantly improved almost all parameters when combined with low and high doses of biotin. For instance, biotin-related enzymes, PSD 95, GFAP, MMP, OPG, IL 17, which did not change even when applying higher doses of biotin, responded well to both magnesium and biotin combinations (MgB1 and MgB2), suggesting that combining biotin with magnesium instead of increasing its dosages might provide more beneficial effects on the process of demyelination and neurobehavioral outcomes. This is consistent with recent prospective MS trials revealing that applying high-dose-biotin as a single agent did not lead to a clear improvement in disability scores [50].

Taken together, we show for the first time that MgB2 was the most effective formulation to improve cognitive scores-neurobehavioral scores, which is associated with improved synaptogenesis, remyelination, bioenergetic mechanisms BDNF levels, and inflammation. To elucidate the underlying neuroprotective and pro-cognitive efficacy of biotin and magnesium in detail, further studies should provide more insights on dose-response relationships of magnesium and biotin. Because biotin and magnesium exert no toxic effects when combined, our data may have a strong translational therapeutic message in cognitive impairment both in progressive and relapsing-remitting MS forms. In conclusion,

increasing the bioavailability of biotin could be a promising neuroprotective and pro-cognitive treatment approach in MS, while bioenergetic enzymes could have critical importance, of which researchers should be aware.

Declarations

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Author Contribution

CO, MT, NS, SC, BE, and BY performed the experiments CO, MT, NS, IHO, SC, BE, BY, and KS analyzed the samples. BY, SPO, and SS wrote a preliminary draft of the manuscript. KS and EK designed experiments, and KS, JK, and EK edited and revised the manuscript. All authors approved the final version of the manuscript

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Data availability

The data will be made available at reasonable request.

Ethics Approval

This study complied with ethical concerns and was approved by the Firat University Animal Research Ethics Committee, Approved number: 257169.

Conflict of Interest

James Komorowski, Sara Perez Ojalvo, and Sarah Sylla are employed by Nutrition 21, LLC, NY, USA. The other authors declare no conflict of interest.

Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

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Figures

Figure 1

Effects of biotin and magnesium biotin (MgB) supplementation on entries to target quadrant (A), probe trial (B), and latency (C) to find the hidden platform from the first day to the fifth day in the Morris water maze task in lysophosphatidylcholine (LPC)-induced demyelination of rats. B1: Biotin 0.9 mg/rat/day; B2: Biotin 9 mg/rat/day; MgB1: Magnesium biotin (Biotin 0.9 mg/rat/day); MgB2: Magnesium biotin (Biotin 9 mg/rat/day).

Figure 2

Effects of biotin and magnesium biotinate (MgB) supplementation on histopathological changes stained by H&E (A) and Luxol fast blue (B). B1: Biotin 0.9 mg/rat/day; B2: Biotin 9 mg/rat/day; MgB1: Magnesium biotinate (Biotin 0.9 mg/rat/day); MgB2: Magnesium biotinate (Biotin 9 mg/rat/day).

Figure 3

Effects of biotin and magnesium biotin (MgB) supplementation on brain interleukin 6 (IL-6, A), interleukin 17 (IL-17, B), tumor necrosis factor- α (TNF- α , C), nuclear factor kappa light chain enhancer of activated B cells (NF- κ B, D), chemokine (C-C motif) ligand 3 (CCL-3, E), chemokine (C-C motif) ligand 5 (CCL-5, F) and chemokine (C-X-C motif) ligand 16 (CXCL-16, G), osteoprotegerin (OPG, H) and matrix metalloproteinase 9 (MMP-9, I) protein levels in lysolecithin (LPC)-induced demyelination of rats. Data are expressed as percent of the control value. Each bar represents the mean and standard error of the mean. Blots were repeated at least 3 times Western blot analysis was performed with actin included to ensure equal protein loading. The data are percentages of the control. a-e: Values within the bars with different superscripts are significantly different (Tukey's post-hoc test, $P < 0.05$). B1: Biotin 0.9 mg/rat/day; B2: Biotin 9 mg/rat/day; MgB1: Magnesium biotin (Biotin 0.9 mg/rat/day); MgB2: Magnesium biotin (Biotin 9 mg/rat/day).

Figure 4

Effects of biotin and magnesium biotin (MgB) supplementation on brain acetyl CoA carboxylase 1 (ACC-1, A), acetyl CoA carboxylase 2 (ACC-2, B), pyruvate carboxylase (PC, C), propionyl-CoA carboxylase (PCC, D), and 3-methylcrotonyl-CoA carboxylase (MCC, E) protein levels in lysolecithin (LPC)-induced demyelination of rats. Data are expressed as a percent of the control value. Each bar represents the mean and standard error of the mean. Blots were repeated at least 3 times. Western blot analysis was performed with actin included ensuring equal protein loading. The data are percentages of the control. a-d: Values within the bars with different superscripts are significantly different (Tukey's post-hoc test, $P < 0.05$). B1: Biotin 0.9 mg/rat/day; B2: Biotin 9 mg/rat/day; MgB1: Magnesium biotin (Biotin 0.9 mg/rat/day); MgB2: Magnesium biotin (Biotin 9 mg/rat/day).

Figure 5

Effects of biotin and magnesium biotinate (MgB) supplementation on brain-derived neurotrophic factor (BDNF, A), growth-associated protein (GAP-43, B), glial fibrillary acidic protein (GFAP, C) and intercellular adhesion molecule 1 (ICAM-1, D) protein levels in lysolecithin (LPC)-induced demyelination of rats. Data are expressed as a percent of the control value. Each bar represents the mean and standard error of the mean. Blots were repeated at least 3 times. Western blot analysis was performed with actin included to ensure equal protein loading. The data are percentages of the control. a-d: Values within the bars with different superscripts are significantly different (Tukey's post-hoc test, $P < 0.05$). B1: Biotin 0.9 mg/rat/day; B2: Biotin 9 mg/rat/day; MgB1: Magnesium biotinate (Biotin 0.9 mg/rat/day); MgB2: Magnesium biotinate (Biotin 9 mg/rat/day).

Figure 6

Effects of biotin and magnesium biotinates (MgB) supplementation on brain Synapsin-I (A), postsynaptic density protein 93 (PSD-93, B) and postsynaptic density protein 95 (PSD-95, C) protein levels in lysophosphatidylcholine (LPC)-induced demyelination of rats. Data are expressed as a percent of the control value. Each bar represents the mean and standard error of the mean. Blots were repeated at least 3 times. Western blot analysis was performed with actin included to ensure equal protein loading. The data are percentages of the control. a-d: Values within the bars with different superscripts are significantly different (Tukey's post-hoc test, $P < 0.05$). B1: Biotin 0.9 mg/rat/day; B2: Biotin 9 mg/rat/day; MgB1: Magnesium biotinates (Biotin 0.9 mg/rat/day); MgB2: Magnesium biotinates (Biotin 9 mg/rat/day).

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