Clinical Nutrition Experimental 23 (2019) 1–14

Contents lists available at ScienceDirect

Clinical Nutrition Experimental

journal homepage: http://www.clinicalnutritionexperimental.com

Methodology

Riboflavin may ameliorate neurological motor disability but not spatial learning and memory impairments in murine model of multiple sclerosis

Mahshid Naghashpour [a, b], Reza Amani [c, d, *], Alireza Sarkaki [e, f], Ata A. Ghadiri [g, h], Alireza Samarbaf-Zadeh [i, j], Sima Jafarirad [b, k], Ahmad Rouhizadeh [g], Azadeh Saki [l]

[a] Abadan School of Medical Sciences, Abadan, Iran
[b] Nutrition and Metabolic Disease Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[c] Food Security Research Center, Department of Clinical Nutrition, School of Nutrition and Food Science, Isfahan University of Medical Sciences, Isfahan, Iran
[d] Diabetes Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[e] Physiology Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[f] Department of Physiology, Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[g] Cell and Molecular Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[h] Department of Immunology, Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[i] Infectious and Tropical Disease Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[j] Department of Virology, Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[k] Department of Nutrition, Faculty of Para-medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[l] Department of Vital Statistics, Faculty of Health, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

ARTICLE INFO

Article history:
Received 24 November 2018
Accepted 1 December 2018
Available online 6 December 2018

SUMMARY

Background & aims: Riboflavin has an important role in myelin formation. This experimental study assesses the interactions between the effects of riboflavin and interferon beta-1a (INF-β1a) on motor disability, spatial learning and memory, and brain-

Abbreviations: ATP, Adenosine triphosphate; BDNF, Brain-derived neurotrophic factor; CFA, Complete Freund's adjuvant; CNS, Central nervous system; EAE, Experimental autoimmune encephalomyelitis; INFβ1A, Interferon beta -1a; MOG$_{35-55}$, Myelin Oligodendrocyte Glycoprotein-$_{35-55}$; MS, Multiple sclerosis; MWM, Morris water maze; PBS, Phosphate buffer saline; PCR, Polymerase chain reaction; PTX, Pertussis toxin; SO1, Sham operated 1; SO2, Sham operated 2; ST1, Sham treatment 1; ST2, Sham treatment 2; T1, Treatment 1; T2, Treatment 2; T3, Treatment 3.

* Corresponding author. Food Security Research Center, Department of Clinical, Nutrition, School of Nutrition and Food Science, Isfahan University of Medical Sciences, Isfahan, Iran. Fax: +98 03136681378.
E-mail addresses: m.naghashpour@abadanums.ac.ir (M. Naghashpour), r_amani@nutr.mui.ac.ir, rezaamani@hotmail.com (R. Amani), sarkaki_a@ajums.ac.ir (A. Sarkaki), ata.ghadiri@hotmail.fr (A.A. Ghadiri), alirezasamarbaf_78@hotmail.com (A. Samarbaf-Zadeh), sjafarirad@gmail.com (S. Jafarirad), arouhizadeh@gmail.com (A. Rouhizadeh), azadehsaki@yahoo.com (A. Saki).

https://doi.org/10.1016/j.yclnex.2018.12.001

Keywords:
Riboflavin
Experimental autoimmune encephalomyelitis
Brain-derived neurotrophic factor
Memory

derived neurotrophic factor (BDNF) in experimental autoimmune encephalomyelitis (EAE).

Methods: In the present research C57BL/6 mice (n = 56) were divided into sham and treatment groups. Riboflavin was administrated (10 mg/kg/day) orally for two weeks alone and/or combined with INF-β1a at 150 IU/g of body weight. After the induction of EAE, the animals were investigated for the clinical signs. Spatial learning and memory were assessed through the standard Morris water maze (MWM). The brain and spinal cord levels of BDNF were studied using real-time polymerase chain reactions and enzyme-linked immunosorbent assay. The data were analyzed using one-way ANOVA, repeated measures, and generalized estimating equations model.

Results: The results in the brain revealed that BDNF mRNA expression ($P < 0.01$) and protein levels ($P < 0.05$) increased in the EAE mice treated with the combination of riboflavin and INF-β1a compared to the treated groups with riboflavin or INF-β1a. Clinical scores were reduced in groups treated with riboflavin compared to other groups. EAE mice treated with riboflavin swam significantly faster in MWM compared to other groups ($P < 0.05$). No significant differences were found between EAE and healthy mice in other spatial learning and memory evaluating variables.

Conclusion: The data highlighted the synergistic role of riboflavin and INF-β1a in improving the disability but not spatial learning and memory mediated by BDNF in EAE.

1. Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) leading a considerable reduction of disabilities in activities of daily and cognitive impairment, which profoundly affects the quality of life [1].

Experimental autoimmune encephalomyelitis (EAE), a commonly employed animal model of MS characterized by enhanced inflammation and neurodegeneration in the CNS, has been shown to recount briefly the hippocampal injury and the synaptic loss with resultant impairment in spatial learning and memory [2]. Brain-derived neurotrophic factor (BDNF) plays a protective role in autoimmune neuroinflammation. BDNF has a neuroprotective role in myelin oligodendrocyte glycoprotein (MOG)-induced EAE [3]. Another level of evidence is provided by the supplementation of exogenous BDNF to the lesion area which enhances the axon protection in the autoimmune demyelination without influencing the immune response leading to decrease the symptoms of EAE and reduce the axonal damage [3]. The molecular mechanism of the relationship between BDNF and axonal damage is not still well defined.

Currently, interferon beta (INF-β) is one of the major medications used for MS therapy. However, there are still several queries regarding its utilization remained to be answered [4].

Riboflavin has an important role in myelin formation. Riboflavin selectively intervenes in the peripheral nerve myelin synthesis. Riboflavin deficiency in young chickens leads to a demyelinating peripheral neuropathy [5]. In riboflavin deficiency, the myelin lipids, cerebrosides, phosphatidylethanolamine, and sphingomyelin, as significant components of the myelin membrane, are reduced. Researchers believe that riboflavin is involved in the metabolism of essential fatty acids in the brain, and that the pathological effects of the riboflavin deficiency is similar to fatty acid deficiency leading to rapid onset of abnormal development and maturation of the brain [6]. The response of CNS to

neurotrophins depends on the intracellular adenosine triphosphate (ATP) level [7]. Ameliorating mitochondrion survival and bio-energy by riboflavin showed its responsiveness to neurotrophins probably by increasing the dendritic sprouting and myelin generation [8]. Given the role of riboflavin as a cofactor for succinate dehydrogenase in the complex II of the mitochondrial respiratory chain producing intracellular ATP [9], the study of the role of riboflavin in BDNF response in MS and EAE seems crucial. In the present study, we aimed to explore whether or not the neuroprotective effect of riboflavin can be mediated through gene expression and protein levels of BDNF in the CNS (i.e. brain and spinal cord) in an EAE model of MS. It was also investigated whether or not riboflavin improves disability, learning, and spatial memory. The answers to these questions could eventually be used to predict the possible role of nutrition therapy in MS patients.

2. Materials and methods

2.1. Animals and experimental groups

Ten-week old female C57BL/6 mice were purchased from Pasteur Institute (Tehran, Iran) and housed in the standard cages under the following controlled conditions: 12 h light/dark cycle, temperature of 20 ± 2 °C, and 50–60% relative humidity with free access to food and water *ad libitium*. Animals were kept for 1 week for acclimation. Measurements to improve welfare assistance and clinical status as well as endpoint criteria were established to minimize suffering and ensure animal welfare. Additionally, food pellets were placed on the bed-cage when the animals began to develop clinical signs to facilitate access to food and water.

The mice (n = 56) were randomly assigned into 7 groups (8 mice in each group) [10] as follows:

1) Sham operated 1 [SO1]; PBS (phosphate buffer saline) as vehicle of pertussis toxin (PTX) as intraperitoneal administration
2) Sham operated 2 [SO2]; PBS + riboflavin as oral gavage
3) Sham treatment 1 [ST1]; EAE + the same volume of water (as vehicle of riboflavin) as oral gavage
4) Sham treatment 2 [ST2]; EAE + sodium acetate buffer (as vehicle of interferon beta -1a) subcutaneously
5) Treatment 1 [T1]; EAE + Interferon beta -1a (INF-β1a) (150 IU/g of body weight [11]) subcutaneously
6) Treatment 2 [T2]; EAE + riboflavin (10 mg/kg of body weight [12,13]) as oral gavage
7) Treatment 3 [T3]; EAE+INF-β1a (150 IU/g of body weight) + riboflavin (10 mg/kg of body weight) as oral gavage

All procedures (Fig. 1) were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals*, (Washington DC: National Academy Press, 1996) and approved by the Ethics Committee for Animal Experimentation at the Ahvaz University of Medical Sciences (AJUMS) (NRC-9208).

2.2. EAE induction

EAE was induced in 10-week old female mice by Hooke Kit™ (Hooke labs, EK2110, Lawrence, MA, USA) according to the manufacturer's instruction as follows: to minimize stress, mice were anesthetized by ether, and then immunization was done through subcutaneous injecting 0.1 ml Myelin Oligodendrocyte Glycoprotein-$_{35\text{-}55}$ ($MOG_{35\text{-}55}$) emulsion (1 mg $MOG_{35\text{-}55}$/mL emulsion) in complete Freund's adjuvant (CFA) to the flanks of each mouse (0.2 ml/mouse). This was followed by the intraperitoneal administration of pertussis toxin (PTX) in phosphate buffer saline (PBS) within 2 and 22–26 h after the injection of the emulsion (0.1 ml/mouse, I.P.) [14]. PTX enhances EAE development by providing additional adjuvant. PTX injection affects cell trafficking, development of Th17 cells [15] and integrity of the blood brain barrier [16]. It is not clear which of these effects influence EAE development. Healthy controlled animals in sham groups were treated with the PBS without PTX and $MOG_{35\text{-}55}$. Treatments started after the first day the clinical signs were observed (days 9–14 post-

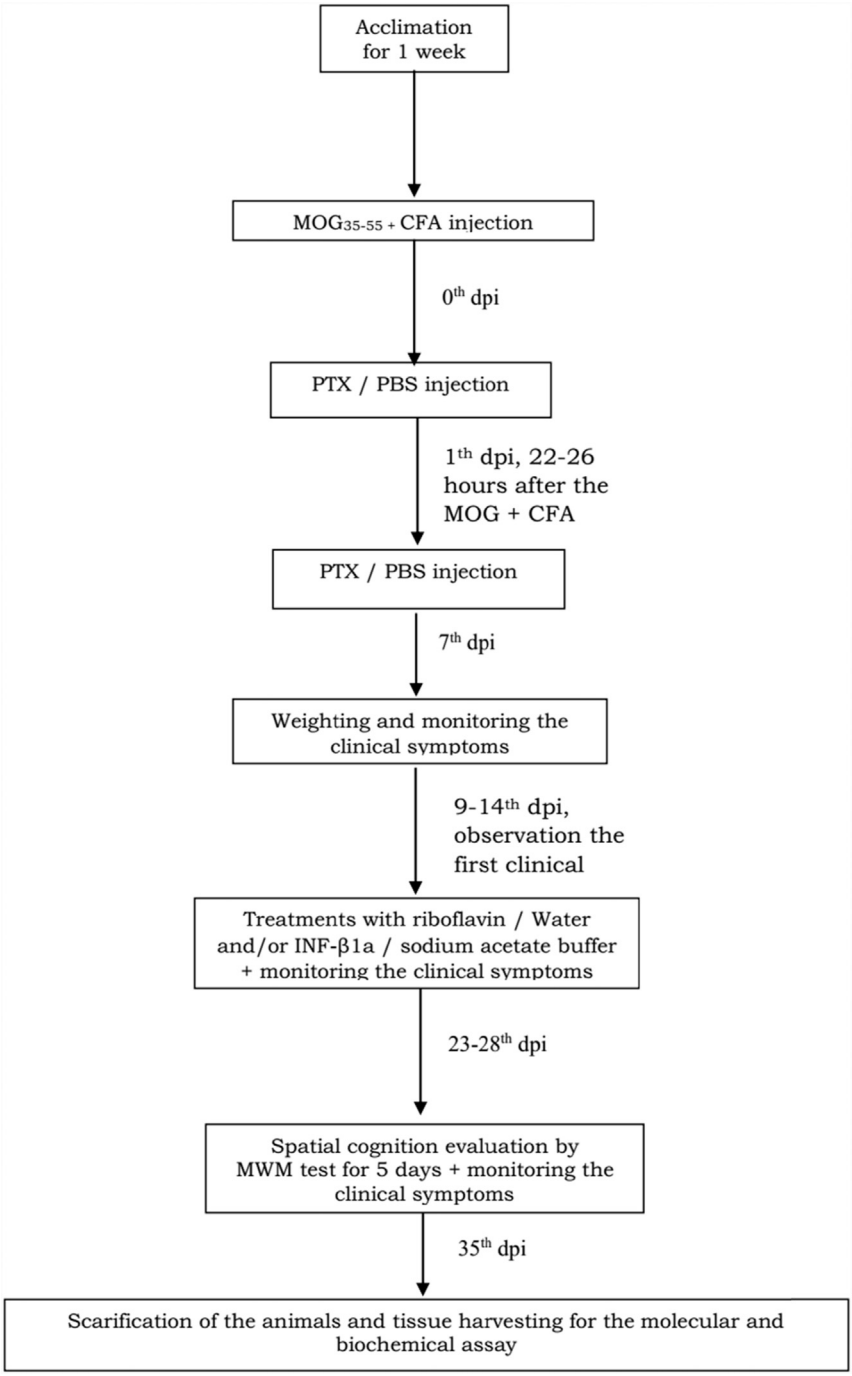

Fig. 1. Illustration of experimental design. MOG_{35-55} (Myelin Oligodendrocyte Glycoprotein-$_{35-55}$); CFA (Complete Freund's adjuvant); PTX (Pertussis toxin); PBS (phosphate buffer saline); INF-β1a (Interferon beta -1a); MWM (Morris water maze).

immunization). Afterwards, the mice were orally gavaged on a daily basis by riboflavin (Puritan's Pride Co., 1233 Montauk Highway Oakdale, NY 11769-9001, United States)/water for two weeks [17] or subcutaneously treated with INF-β1a (RECIGEN®, CinnaGenCo., Tehran, Iran)/sodium acetate buffer for two weeks [4]. In the group [T3], the mice received a combination of riboflavin and interferon beta -1a (Fig. 1).

2.3. Clinical evaluation

The mice were checked for clinical symptoms of the EAE each day starting on the day before immunization and 7th to 35th days post-immunization (dpi). The animal weight and clinical scores were monitored every day as previously described [18]. The scores were defined as follows: 0 = no clinical signs, 0.5 = partial tail paralysis, 1.0 = complete tail paralysis, 1.5 = complete tail paralysis and discrete hind limb weakness, 2 = tail paralysis and hind-limb weakness, 2.5 = unilateral hind limb paralysis, 3 = complete hind limb paralysis, 3.5 = hind limb paralysis and forelimb weakness, 4.0 = complete paralysis (tetraplegia), and 5.0 = moribund or dead.

2.4. Tissue harvesting and sectioning

After 35 days, the mice were deeply anesthetized with ketamine and xylazine (100 mg/kg) as detailed by the previous study [19]. The animals were then sacrificed and their whole brain and spinal cord were removed and rinsed in ice-cold PBS (0.02 mol/L, pH 7.0–7.2) to remove the excess blood thoroughly. The specimens were placed on an ice-cold surface, cut in half, and weighed. The brain hemispheres and spinal cord were snap-frozen in liquid nitrogen and stored at −80 °C for further use [20].

2.5. Molecular and biochemical assays

In order to measure all biochemical and molecular parameters in the same region, the whole brain and spinal cord were removed [21].

2.6. RNA preparation and cDNA synthesis

The total cellular RNA from the whole brain and the spinal cord was extracted using the RNA extraction system of Qiagen® according to manufacturer's protocol (RNeasy® Lipid Tissue Mini Kit, Hilden, Germany). cDNA was synthesized from ≤5 μg of total RNA via the oligo $(dt)_{12\text{-}18}$ primers (Invitrogen™by Life Technologies, Frankfurter, Germany) according to the manufacturer's protocol. cDNA samples were stored at −20 °C for further use.

To check the quality and quantity of synthesized cDNA, PCR reactions were performed as previously described [22] using a thermocycler (Bio-Rad, Hercules, California 94547, USA) in a final volume of 50 ml. Aliquots of PCR reaction products (approximately 20 ml), previously normalized to give equivalent amounts of the GAPDH control product in all samples, were electrophoresed on 0.8% agarose gels. The gels were visualized under UV light, and their images were captured using the gel documentation system (Uvitec; Cambridge, United Kingdom). Synthesized cDNA bands were 500 bp.

2.7. Quantitative real-time PCR

mRNA was used for determining the BDNF and β-actin (as housekeeping gen) gene expression via a Step One fluorescence-based Real-time PCR system (Applied Biosystems, Foster City, CA, USA) by presence of mouse TaqMan®probe (Applied Biosystems) according to the manufacturer's instructions to determine BDNF transcripts in the brain and spinal. The total volume reaction was 10 μl applying 5 μl of Taqman gene expression Master Mix reagent (Applied Biosystems, Foster City, CA, USA), 0.5 μl of cDNA, 0.5 μl of BDNF TaqMan® probe, and 4 μl of RNase free water. For each gene, the samples were carried out as duplicates, and the reactions were repeated three times. The PCR for the BDNF and the β-actin were performed on the same reaction plate. Thermal cycling conditions included 2 min at 50 °C,

10 min at 95 °C, 45 cycles for 15 s at 95 °C, and 1 min at 60 °C. The assay was performed in duplicate for each sample [23]. The amount of targets, normalized to an endogenous reference, was defined by the Ct (threshold cycle) methods for comparing the relative expression ratio in real-time PCR among the treatment and the control groups [24]. The average and standard error mean (SEM) of the relative expression values were calculated and the significance of the change was tested using the Student's t-test with unequal variance. A P-value of <0.05 was considered significant.

2.8. Enzyme-linked immunosorbent assay (ELISA)

Brain and spinal cord tissues were homogenized with a digital teflon homogenizer (model Silent Crusher M; Heidolph Co., Schwabach, Germany) on ice at 4000 rpm for 30 s in a lysis buffer containing 137 mM NaCl, 20 mM Tris–HCl (pH 8.0), 1% NP40 (Sigma–Aldrich®, St. Louis, MO, USA), 10% glycerol, 0.5 mM sodium orthovanadate (Sigma–Aldrich®), and complete protease inhibitor cocktail (Sigma–Aldrich®) containing 104 mM AEBSF, 80 μM Aprotinin, 4 mM Bestatin, 1.4 mM E–64, 2 mM Leupeptin, and 1.5 mMPepstatin, then centrifuged at 2464 g-force at 4 °C for 20 min. The supernatants were removed and diluted by adding 4 volumes of Dulbecco's phosphate buffer saline (DPBS). Aliquots were further processed at room temperature by acidification with 1N HCl (pH < 3) for 15 min, neutralized with 1N NaOH (pH 7–8), and then used for ELISA analysis.

Levels of the BDNF were quantified by the BDNF EMAX® Immuno Assay System (Promega, Madison, USA) according to the manufacturer's instructions [25]. All reactions were performed twice. The absorbance was measured at 450 nm by ELISA reader (BioTek, ELx800, Dickinson, Texas, USA) [26]. BDNF concentrations were expressed as picograms of BDNF per milligram of tissue (pg/mg tissue) as formerly reported [26].

2.9. Spatial cognition evaluation

Spatial learning and memory were assessed through the standard Morris water maze (MWM) (Maze Router V3.1, TechniqAzma Co., Tabriz, Iran) consisting of a circular tank (120 cm diameter, and 60 cm height) filled with opaque water. The circular tank was divided into four quadrants delimiting separate zones. The escape platform (12 cm diameter) was located in the north-east quadrant in a fixed position 2 cm under the water surface; this quadrant was defined as the target zone. Visual cues were placed in each of the four arbitrarily defined quadrants of the tank. During 4 days of initial training, the mice were put in the water maze facing the wall in one of the four equal quadrants labeled north, east, south, and west which were randomly changed in every trial. The spatial cues for reference around the pool were maintained constant throughout the experiment. The mice were given four training trials per day. Each trial lasted until the mouse found the platform or swam for a maximum of 60 s. Regardless of success or failure to reach the hidden platform, all mice were given an additional 30 s on the platform and then were placed in its heated cage until the next trial. The spatial learning variables were swimming distance (cm), latency to find the hidden platform (s), and average swimming speed (cm/s), recorded by the computer-controlled Smart Video tracking system (Technique Azma Co., Tabriz, Iran). On the fifth day (probe trial), the platforms were removed from the target zone, the mice were placed in the tank from the region apposite of target quadrant, and were allowed to swim for 60 s to search for the submerged platform. The spatial memory variables including the spent time in the target zone (s) and the percentage of the time spent in the target zone (%) were recorded [2].

2.10. Statistical consideration

For the biochemical, the Real-time PCR, and the clinical observations assays data were analyzed using ANOVA followed by LSD's and Bonferroni's post-Hoc tests. Repeated measures of ANOVA were applied to assess the differences and variance amongst different experimental groups in Morris Water Maze. GEE (Generalized Estimating Equations) model was used to compare the clinical scores among the experimental groups and the evaluation of interaction and synergistic effects among riboflavin and interferon beta -1a. The data were analyzed by statistical package for social sciences (SPSS) version 21 (SPSS Inc., Chicago, USA). Numerical data were normally distributed, hence, they

were expressed as the mean ± standard error of the mean (SEM). All mean differences were considered significant if $P < 0.05$.

3. Results

3.1. BDNF gene expression and its protein level in the brain and spinal cord

Real-time PCR analysis of mRNA expression for BDNF was conducted on whole brain and spinal cord samples obtained from the seven experimental groups. The BDNF mRNA expression was assessed in parallel with that of the housekeeping gene β-actin. The results in the brain analyzed by one way ANOVA followed by LSD's post-Hoc test revealed that BDNF mRNA expression increased in the T3 EAE group compared to SO1 (95% confidence interval, CI: 4.2–15.6), SO2 (95% CI: 4.2–15.6), ST1 (95% CI: 4.6–15.2), ST2 (95% CI: 4.4–15.4), T1 (95% CI: 3–14), and T2 (95% CI: 4.1–14.7) groups. In addition, one way ANOVA followed by Bonferroni's post - Hoc test revealed similar results ($P < 0.01$, Fig. 2a). The results showed that animals that received the combination of INF-β1a and riboflavin have ten times more BDNF mRNA compared to sham, seven times more BDNF mRNA compared to T2, and 4.5 times more BDNF mRNA compared to T1 groups. Based on our model of MS induction [25], we hypothesized that BDNF protein level increased in the whole brain. To test our hypothesis, we assessed the presence of BDNF in the whole brain of the healthy controls and EAE animals. One way ANOVA followed by LSD's post - Hoc test showed a markedly increase in BDNF protein levels in the whole brain of T3 (2463.4 ± 832.8 pg/mg tissue) EAE animals compared to ST2 (1055.3 ± 163 pg/mg tissue; 95% CI: 13.9–2802.2), T1 (945 ± 148.1 pg/mg tissue; 95% CI: 63.5–2973.1), and T2 (837.6 ± 313.7 pg/mg tissue; 95% CI: 231.7–3020) EAE mice ($P < 0.05$, Fig. 2c). But one way ANOVA followed by Bonferroni's post-Hoc test showed no significant difference between groups in terms of BDNF protein level in the whole brain.

Moreover, the real time PCR analysis conducted by one way ANOVA followed by LSD's post- Hoc test revealed a significant increase in BDNF mRNA expression in the spinal cord of the T3 group compared to SO1 (95% CI: 2.8–7.5), SO2 (95% CI: 2.8–7.5), ST1 (95% CI: 3–7.4), ST2 (95% CI: 2.9–7.4), T1 (95% CI: 1.1–6.1), and T2 (95% CI: 1.1–5.5) groups. In addition, one way ANOVA followed by Bonferroni's post-Hoc test showed the similar results ($P < 0.001$, Fig. 2b). The results showed that animals that received the combination of INF-β1a and riboflavin have six times more BDNF mRNA in the spinal cord compared to sham and two times more BDNF mRNA in the spinal cord compared to other treatment groups. ELISA analysis of BDNF expression was conducted on the spinal cord of animals. T1 and T2 groups' spinal cord contained 289.3 ± 76.4 and 223 ± 69.5 pg/mg tissues, respectively. One way ANOVA followed by LSD's post -Hoc test revealed that animals in T1 group showed a significant enhancement of BDNF levels compared to SO1 (17.4 ± 17.4 pg/mg tissues; 95% CI: 119.9–423.9), SO2 (3.8 ± 2 pg/mg tissues; 95% CI: 155.9–415.1), ST1 (96.5 ± 31 pg/mg tissues; 95% CI: 67.3–318.3), ST2 (40.6 ± 23.3 pg/mg tissues; 95% CI: 113.8–383.6), and T3 (8.5 ± 5 pg/mg tissues; 95% CI: 155.3–406.3) groups. Animals in T2 group showed a significant enhancement of BDNF levels compared to SO1 (95% CI: 57.1–354.1), SO2 (95% CI: 93.7–344.7), ST1 (95% CI: 5.3–247.8), ST2 (95% CI: 51.4–313.4), and T3 (95% CI: 93.3–335.8) groups. In addition, one-way ANOVA followed by Bonferroni's post-Hoc test showed similar results ($P < 0.001$, Fig. 2d).

3.2. Riboflavin supplementation ameliorates neurological disability but not cognitive impairment in EAE mice

All animals in the EAE groups were assessed for the neurological disability from 7th to 35th day after immunization according to a global neurological disability assessment tool [18]. By scoring animals in each group daily, means of clinical scores calculated. Daily clinical scores reduced significantly in both riboflavin treated groups (T2 and T3) compared to other groups at the effector phase at 26–35 dpi (1.1 ± 0.02 for both groups) and chronic phase at 30 to 35 dpi (1.20 ± 0.03 and 1 ± 0.01, respectively) of the disease ($P < 0.01$, Fig. 3). GEE model was used for the evaluation of the interaction and synergistic effects between riboflavin and INF-β1a on clinical scores. This model showed that the INF-β1a did not

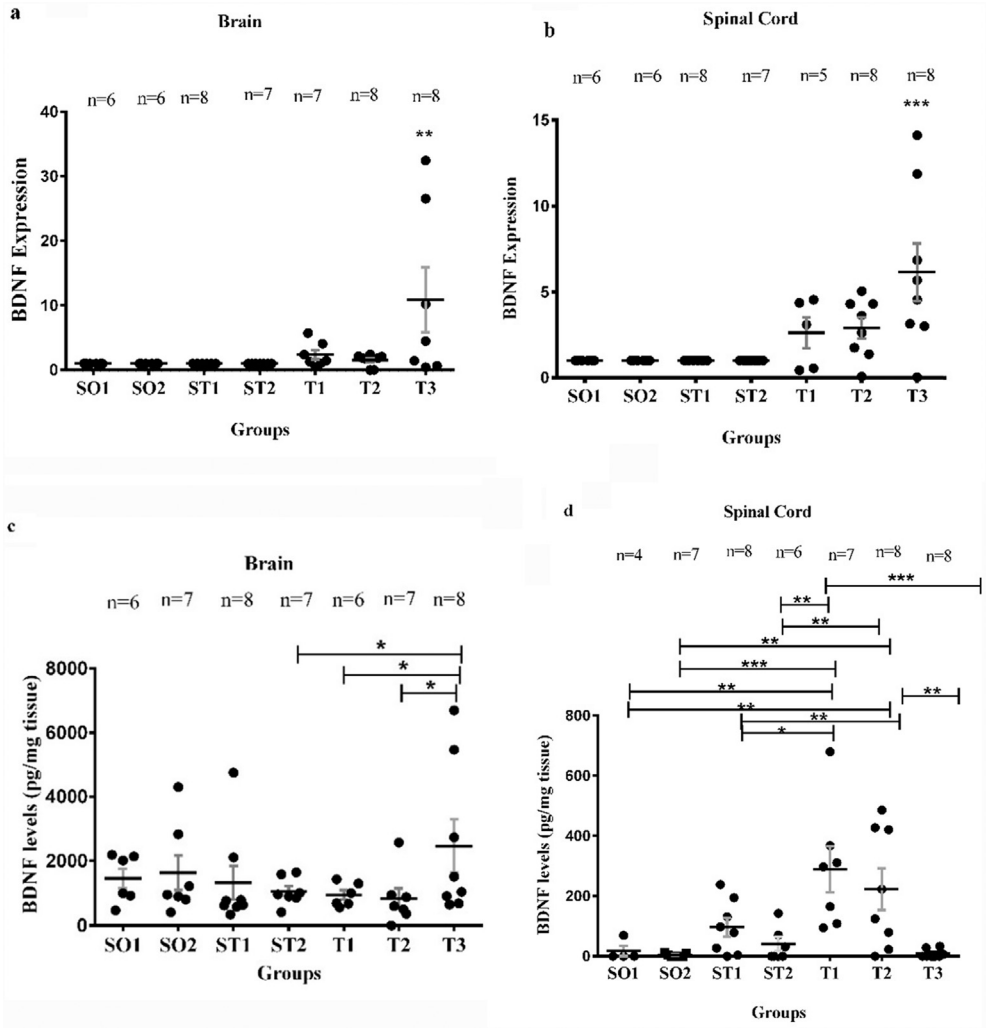

Fig. 2. Gene expression and protein levels of BDNF in the brain and spinal cord of study groups. (a) Animals in the T3 group increased significantly BDNF expression in the whole brain compared to sham operated, sham treated, and treatment groups (**$P < 0.01$). (b) EAE mice in T3 group showed a significant increase in BDNF gene expression in the spinal cord compared to sham operated, sham treated, and treatment groups (***$P < 0.001$). (c) Animals in T3 group showed a significant increase in BDNF protein levels in the whole brain compared to ST2, T1, and T2 EAE mice (*$P < 0.05$). (d) Animals in SO1 and SO2 groups, and EAE animals in ST1, ST2, and T3 groups showed a significant decrease in BDNF expression in the spinal cord compared to EAE mice in T1 and T2 groups (*$P < 0.05$,**$P < 0.01$, ***$P < 0.001$). The data are given as mean ± SEM analyzed by one way ANOVA and LSD's and Bonferroni's post -Hoc tests. The results are shown as pg/mg tissue of BDNF. SO1 (sham operated 1, Healthy+PBS); SO2 (sham operated 2, healthy + PBS); ST1 (sham treatment 1, EAE+Water); ST2 (sham treatment 2, EAE+sodium acetate buffer); T1 (treatment 1, EAE + INFβ1A); T2 (treatment 2, EAE+riboflavin); T3 (Treatment 3, EAE + INFβ1A+riboflavin); BDNF (brain-derived neurotrophic factor). The experiments were repeated three times in duplicates.

affect the clinical scores ($p = 0.249$). Riboflavin and the combination of the riboflavin and INF-β1a significantly decreased the clinical scores ($p = 0.000$). Results illustrated that the simultaneous administration of the INF-β1a and riboflavin has more improving effects on neurological disability rather than INF-β1a administration per se (Fig. 3).

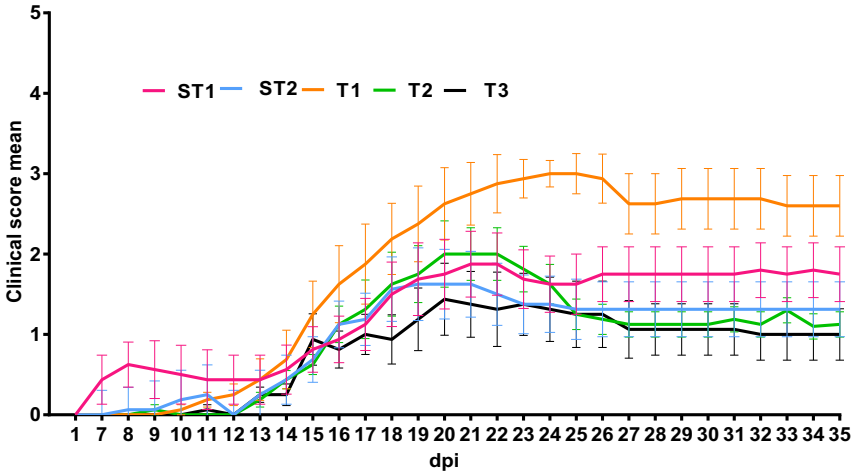

Fig. 3. Clinical scores of EAE mice. Differences were observed between the EAE mice in different groups from 11 to 15 dpi ($P < 0.05$). ST1 (sham treatment 1, EAE+Water); ST2 (sham treatment 2, EAE+sodium acetate buffer); T1 (treatment 1, EAE + INFβ1A); T2 (treatment 2, EAE+riboflavin); T3 (Treatment 3, EAE + INFβ1A+riboflavin).

Morris water maze data revealed that mice with EAE performed similarly to healthy matched to controls in terms of latency (Fig. 4) and length (swimming distance) (data have not been shown) to find the hidden platform. No significant differences were seen between EAE and healthy normal mice in swimming latency and length of finding the hidden platform. Latency trended to a decrease in all study groups, but this decrease was not statistically significant (Fig. 4).

T2 mice treated with riboflavin swam significantly faster compared to ST2 ($P = 0.025$) and T1 mice ($P = 0.024$). Moreover, T3 mice treated with both riboflavin and INF-β1a swam significantly faster than ST1 mice at day 4 of the training ($P = 0.045$) (Fig. 5).

Percentage of time spent in the target quadrant measured for evaluating the spatial memory which showed no significant difference among the groups (Fig. 6).

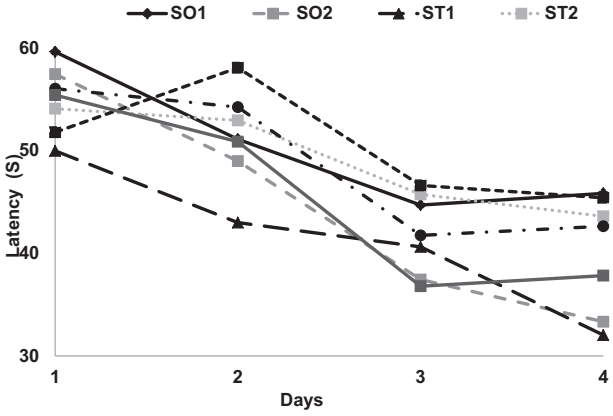

Fig. 4. Latency to find the hidden platform (s) in spatial learning in Morris water maze by mice treated with riboflavin and/or INFβ-1A. All data are shown as Mean ± SEM (N = 8). Repeated Measure test followed by Post-Hoc LSD tests demonstrated that during the 4 days of trials, the latency to the plate obtain showed no significant difference among the groups. SO1 (sham operated 1, PBS); SO2 (sham operated 2, PBS+riboflavin); ST1 (sham treatment 1, EAE+Water); ST2 (sham treatment 2, EAE+sodium acetate buffer); T1 (treatment 1, EAE + INF-β1a); T2 (treatment 2, EAE+riboflavin); T3 (Treatment 3, EAE + INF-β1a+riboflavin).

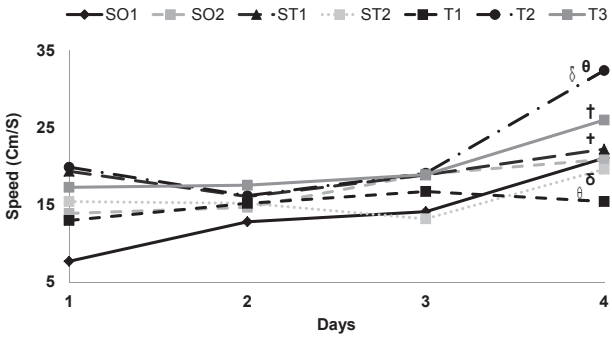

Fig. 5. Swimming speed to find the hidden platform (cm/s) in spatial learning in Morris water maze by mice treated with riboflavin and/or INFβ-1A. All data are shown as Mean ± SEM (N = 8). Repeated Measure test followed by Post-Hoc LSD tests showed that during the 4 days of trials, the speed to obtain the hidden plate indicated significant differences in all groups. a) There was no significant difference between SO1, ST1, and ST2 groups. b) There was no significant difference between SO2, ST1, and T2 groups. c) T2 mice treated with riboflavin swam significantly faster than ST2 and T1 mice. d) T3 mice treated with both riboflavin and INF-β1a swam significantly faster than ST1 mice on the 4th day of the trial. (†$P = 0.045$, δ $P = 0.025$, θ $P = 0.024$). SO1 (sham operated 1, PBS); SO2 (sham operated 2, PBS+ riboflavin); ST1 (sham treatment 1, EAE+Water); ST2 (sham treatment 2, EAE+sodium acetate buffer); T1 (treatment 1, EAE + INF-β1a); T2 (treatment 2, EAE+riboflavin); T3 (Treatment 3, EAE + INF-β1a+riboflavin).

4. Discussion

The results of this study state that riboflavin has beneficial effects on neurological motor disability but not on the improvement of spatial learning and memory impairments in an EAE model of MS. Riboflavin diminishes the clinical severity of EAE and has an inhibitory effect on EAE courses. The inhibitory effects of riboflavin start at the effector phase of the disease and continue to the chronic phase. These effects of riboflavin are associated with the modulation of the neurotrophic activity. This finding might indicate that the potential therapeutic effects of riboflavin are mainly exerted through stabilizing the disease course. We also evaluated the effect of riboflavin and INF-β1a combination on the neurologic disability. The purpose of this combination was to

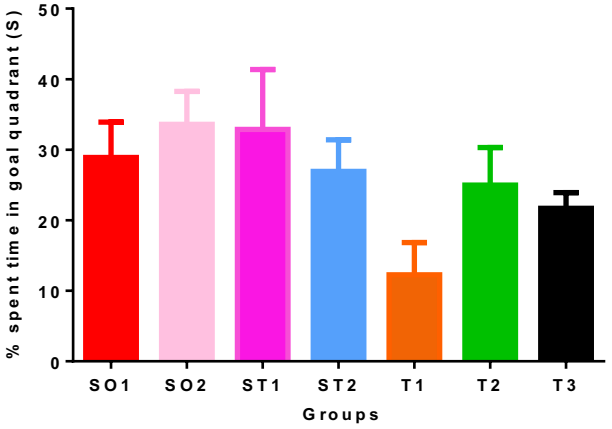

Fig. 6. Performance in reference memory version of the Morris water maze by mice treated with riboflavin and/or INFβ1A. All data are shown as Mean ± SEM (N = 8). ANOVA test followed by Post-hoc LSD tests showed that no significant difference was found between the groups in terms of spatial memory variable (percentage of spent time in the goal quadrant). SO1 (sham operated 1, PBS); SO2 (sham operated 2, PBS+riboflavin); ST1 (sham treatment 1, EAE+Water); ST2 (sham treatment 2, EAE+sodium acetate buffer); T1 (treatment 1, EAE + INF-β1a); T2 (treatment 2, EAE+riboflavin); T3 (Treatment 3, EAE + INF-β1a+riboflavin).

avoid depriving animal from possible disease modifying therapy and to evaluate interactions (antagonistic or synergistic effects) with riboflavin supplement. This study demonstrated that riboflavin has synergistic effects with INF-β1a on EAE disease. We have previously showed that although supplementation with riboflavin (10 mg/day) for six months reduces the expanded disability status scale (EDSS) score significantly in patients with MS, this reduction also occurred in the MS patients taking placebo. The decrease rate was non-significantly higher in the riboflavin than in the placebo group (26.4% vs. 15.4% for MS patients taking placebo). To the best of our knowledge, that study was the first to assess the effect of riboflavin on disability in MS patients [27]. Interestingly, Ogunleye & Odutuga [6] showed that myelin lipids, cerebrosides, sphingomyelin, as well as phosphatidyl ethanolamines, as essential components of the myelin sheath are considerably reduced in riboflavin deficit rats. Moreover, selective injury to peripheral nerve trunks, with relative sparing of spinal nerve roots and distal nerve branches to muscle and skin, had been observed in the young chickens with riboflavin deficiency [5].

Suppression of the clinical signs of EAE by riboflavin was accompanied by an increase in gene expression and protein levels of BDNF in the brain and spinal cord, suggesting the positive effects of riboflavin on the disease process which probably intermediated by increased gene expression and protein levels of BDNF. As we know, BDNF is one of the most abundant neurotrophins and is widely distributed in the CNS. In EAE and MS, T cells in the vicinity of demyelinating lesions express BDNF, suggesting the neuroinflammatory reaction and limiting brain damage [21]. Our results showed an enhancement of BDNF content in the brain and spinal cord of EAE mice receiving riboflavin. Since, ATP is necessary for the release of BDNF from the CNS neurons [7], perhaps riboflavin can improve the response to BDNF mediated by facilitating mitochondrial bio-energetics increasing dendritic sprouting and myelin generation [8]. There are several ways to stimulate mitochondrial function. Much has been learned from utilizing metabolic vitamin/mineral coenzymes and energy substrates in mitochondrial disorders. Riboflavin-5 phosphate improves mitochondrial function and reduces excitotoxicity [28].

In this study, the EAE mice treated with INF-β1a had worse disease than other groups in term of clinical score means. Furthermore, the speed of animals in MWM test decreased significantly compared to the other groups. However, the protein levels of BDNF increased in the spinal cord of mice receiving IFNβ-1a or riboflavin alone but not in the mice receiving the combination of IFNβ-1a and riboflavin as compared to other groups. This is an unexpected result that opposed the conclusion of synergistic beneficial effect with riboflavin. IFN-β effects are specific for immune subsets [29], possibly explaining why IFNβ-1a therapy is effective in increasing the levels of BDNF in the spinal cord but not the brain. Moreover, a study showed that mice receiving IFN-β had reduced apoptosis and increased growth promoting factors including BDNF [30]. However, more studies are needed to confirm the role of BDNF in the regulation of response to IFN-β therapy. One hypothesis may explain the more severe diseases in EAE mice treated with IFNβ-1a. Some experiments detected that mice treated with IFN-β showed severe acute clinical symptoms which were significantly increased in comparison to the PBS treated mice. Mice with TH17-EAE did not respond to IFN-β treatment. Additionally, IFN-β had pro-inflammatory role in TH17 induced EAE. Therefore, not only would the IFN-β treatment be ineffective but also it could worsen the symptoms [4].

Furthermore, our results study demonstrated that riboflavin had no effect on the improvement of memory impairments in EAE and control mice. Intriguingly, recovery of speed in MWM test was seen when EAE mice took riboflavin and/or both riboflavin and IFNβ-1a. It is reminded that for ethical purposes, only mice were included in MWM test whose clinical scores were lower than 3, making it difficult to determine whether riboflavin improves spatial learning and memory during the chronic phase of EAE. Moreover, it should be considered that motor activity as shown by swim speed might be affected by changes in motor disability in EAE mice. This finding has been confirmed in a similar study by Kim et al. [2] who reported that spatial learning deficits were associated with motor disability in EAE mice.

In this study, the SO2, T2, and T3 groups received riboflavin at supraphysiological dose (10 mg/kg of body weight) during two weeks. No changes in the overall health aspects of the healthy young rats treated by riboflavin supplement with similar dose were witnessed. Moreover, the riboflavin supplementation with 10 mg/kg of body weight did not provoke any side effects. However, it is recommended

that MS patients be supported by a possible protective function against MS risks via a daily multivitamin supplement containing almost 10 mg riboflavin [13]. These results are in agreement with the fact that riboflavin is a safe agent and has been approved for clinical usage.

The limitation of this study is the lack of control group for EAE mice receiving the combination of riboflavin and INF-β1a. Further studies are suggested to assess that supersession of EAE induction with riboflavin administration is in parallel with immunization and also to evaluate the preventive role of riboflavin in MS. It is also suggested that gene expression and the serum concentrations of IL-17 should be assessed. In addition, the estimation of interferon beta might have predictive value as a biomarker to assess the response to interferon beta treatment. Likewise, the levels of glucocorticoids and other inflammatory mediators such as IL-6, IFN-γ, and TNF -α (tumor necrosis factor- α) at the time of immunization might be useful in clarifying the mechanism of the observed results in this study. Histological evaluation is important in the treatment of riboflavin. We would suggest to assess the degree of inflammation and demyelination in spinal cord and brain. The findings of our study demonstrate a novel approach in the application of riboflavin supplementation in EAE and warrants further studies which including the investigation of possible role of inflammatory mediators in the mechanism of riboflavin in MS treatment.

In summary, these findings propose the fact that the combination of riboflavin and INF-β1a may be beneficial in reducing the deleterious effects of neurological disability on EAE model of MS. Moreover, they showed that the supplementation of riboflavin increases the gene expression and protein levels of BDNF in the brain and spinal cord after EAE induction, suggesting that BDNF may mediate the beneficial effects of riboflavin on neurological disability.

Authorship

Mahshid Naghashpour formulated the research questions, designed the study and carried it out. Reza Amani and Alireza Sarkaki contributed as supervisors, formulated the research questions, and designed the study. Ata A. Ghadiri contributed in immunologic consultant. Alireza Samarbafzadeh contributed in genetic consultant. Sima Jafarirad contributed in nutritional and biochemical consultant. Ahmad Rouhizadeh contributed in laboratory procedures, and Azadeh Saki contributed in statistical consultant. All authors contributed in writing the article and approved it before submission.

Funding sources

This work was supported by the Vice-Chancellor for Research, Ahvaz Jundishapur University of Medical Sciences (Grant number: NRC-9208). Also, CinnaGen® Co. provided the RECIGEN®. Vice-Chancellor for Research, Ahvaz Jundishapur University of Medical Sciences and CinnaGen® Co had no role in the design, analysis or writing of this article.

Conflicts of interest

None of the authors has any conflicts of interest to declare.

Acknowledgments

This manuscript was extracted as a part of Mahshid Naghashpour's Ph.D. thesis. This study was supported by the grant of Vice-Chancellor for Research Affairs, Ahvaz Jundishapur University of Medical Sciences (Grant number: NRC-9208). We wish to thank Dr. Mohammadreza Rashidi Nooshabadi for his help in providing riboflavin supplement.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yclnex.2018.12.001.

References

[1] Henze T, Feneberg W, Flachenecker P, Seidel D, Albrecht H, Starck M, et al. New aspects of symptomatic MS treatment: Part 6 - cognitive dysfunction and rehabilitation. Nervenarzt 2017. https://doi.org/10.1007/s00115-017-0443-7.
[2] Kim do Y, Hao J, Liu R, Turner G, Shi FD, Rho JM. Inflammation-mediated memory dysfunction and effects of a ketogenic diet in a murine model of multiple sclerosis. PLoS One 2012;7, e35476. https://doi.org/10.1371/journal.pone.0035476.
[3] Linker RA, Lee DH, Demir S, Wiese S, Kruse N, Siglienti I, et al. Functional role of brain-derived neurotrophic factor in neuroprotective autoimmunity: therapeutic implications in a model of multiple sclerosis. Brain 2010;133:2248–63. https://doi.org/10.1093/brain/awq179.
[4] Axtell RC, de Jong BA, Boniface K, van der Voort LF, Bhat R, De Sarno P, et al. T helper type 1 and 17 cells determine efficacy of interferon-beta in multiple sclerosis and experimental encephalomyelitis. Nat Med 2010;16:406–12. https://doi.org/10.1038/nm.2110.
[5] Cai Z, Blumbergs PC, Finnie JW, Manavis J, Thompson PD. Selective vulnerability of peripheral nerves in avian riboflavin deficiency demyelinating polyneuropathy. Vet Pathol 2009;46:88–96. https://doi.org/10.1354/vp.46-1-88.
[6] Ogunleye AJ, Odutuga AA. The effect of riboflavin deficiency on cerebrum and cerebellum of developing rat brain. J Nutr Sci Vitaminol (Tokyo) 1989;35:193–7. http://www.ncbi.nlm.nih.gov/pubmed/2760691.
[7] Coull JA, Beggs S, Boudreau D, Boivin D, Tsuda M, Inoue K, et al. BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. Nature 2005;438:1017–21. https://doi.org/10.1038/nature04223.
[8] Reese D, Shivapour ET, Wahls TL, Dudley-Javoroski SD, Shields R. Neuromuscular electrical stimulation and dietary interventions to reduce oxidative stress in a secondary progressive multiple sclerosis patient leads to marked gains in function: a case report. Cases J 2009;2:7601. https://doi.org/10.4076/1757-1626-2-7601.
[9] Cicek G, Schiltz E, Hess D, Staiger J, Brandsch R. Analysis of mitochondrial antigens reveals inner membrane succinate dehydrogenase flavoprotein subunit as autoantigen to antibodies in anti-M7 sera. Clin Exp Immunol 2002;128:83–7. http://www.ncbi.nlm.nih.gov/pubmed/11982594.
[10] Deshmukh VA, Tardif V, Lyssiotis CA, Green CC, Kerman B, Kim HJ, et al. A regenerative approach to the treatment of multiple sclerosis. Nature 2013;502:327–32. https://doi.org/10.1038/nature12647.
[11] Martinez PCI M A, Vaello ML, Garcia-Merino A. Anne M. Pilaro.Toxicologist's review. PLA1998.
[12] Feitoza FCVL. The response of young and adult rats to the riboflavin supplementation. Intervent J Braz Arch Biol Technol 2010;53:855–60.
[13] Kurn S, Shoorn SH. Integrated medicine for neurological disorders Albuquerque New Mexico. Health Press NA Inc; 2008.
[14] Bittner S, Afzali AM, Wiendl H, Meuth SG. Myelin oligodendrocyte glycoprotein (MOG35-55) induced experimental autoimmune encephalomyelitis (EAE) in C57BL/6 mice. J Vis Exp 2014. https://doi.org/10.3791/51275. 10.3791/51275.
[15] Chen X, Howard OM, Oppenheim JJ. Pertussis toxin by inducing IL-6 promotes the generation of IL-17-producing CD4 cells. J Immunol 2007;178:6123–9. http://www.ncbi.nlm.nih.gov/pubmed/17475838.
[16] Lu C, Diehl SA, Noubade R, Ledoux J, Nelson MT, Spach K, et al. Endothelial histamine H1 receptor signaling reduces blood-brain barrier permeability and susceptibility to autoimmune encephalomyelitis. Proc Natl Acad Sci USA 2010;107:18967–72. https://doi.org/10.1073/pnas.1008816107.
[17] França CF, Vianna L. The response of young and adult rats to the riboflavin supplementation. Braz Arch Biol Technol 2010;53:855–60.
[18] Soleimani M, Jameie SB, Barati M, Mehdizadeh M, Kerdari M. Effects of coenzyme Q10 on the ratio of TH1/TH2 in experimental autoimmune encephalomyelitis model of multiple sclerosis in C57BL/6. Iran Biomed J 2014;18:203–11. http://www.ncbi.nlm.nih.gov/pubmed/25326018.
[19] Xu Q, Ming Z, Dart AM, Du XJ. Optimizing dosage of ketamine and xylazine in murine echocardiography. Clin Exp Pharmacol Physiol 2007;34:499–507. https://doi.org/10.1111/j.1440-1681.2007.04601.x.
[20] Datta SC, Opp MR. Lipopolysaccharide-induced increases in cytokines in discrete mouse brain regions are detectable using Luminex xMAP technology. J Neurosci Methods 2008;175:119–24. https://doi.org/10.1016/j.jneumeth.2008.08.007.
[21] Ogonovszky H, Berkes I, Kumagai S, Kaneko T, Tahara S, Goto S, et al. The effects of moderate-, strenuous- and over-training on oxidative stress markers, DNA repair, and memory, in rat brain. Neurochem Int 2005;46:635–40. https://doi.org/10.1016/j.neuint.2005.02.009.
[22] Teixeira SA, Castro GM, Papes F, Martins ML, Rogerio F, Langone F, et al. Expression and activity of nitric oxide synthase isoforms in rat brain during the development of experimental allergic encephalomyelitis. Brain Res Mol Brain Res 2002;99:17–25. http://www.ncbi.nlm.nih.gov/pubmed/11869804.
[23] Nosrat IV, Margolskee RF, Nosrat CA. Targeted taste cell-specific overexpression of brain-derived neurotrophic factor in adult taste buds elevates phosphorylated TrkB protein levels in taste cells, increases taste bud size, and promotes gustatory innervation. J Biol Chem 2012;287:16791–800. https://doi.org/10.1074/jbc.M111.328476.
[24] Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:e45. http://www.ncbi.nlm.nih.gov/pubmed/11328886.
[25] Zhu W, Frost EE, Begum F, Vora P, Au K, Gong Y, et al. The role of dorsal root ganglia activation and brain-derived neurotrophic factor in multiple sclerosis. J Cell Mol Med 2012;16:1856–65. https://doi.org/10.1111/j.1582-4934.2011.01481.x.
[26] Kheirvari S, Uezu K, Yamamoto S, Nakaya Y. High-dose dietary supplementation of vitamin A induces brain-derived neurotrophic factor and nerve growth factor production in mice with simultaneous deficiency of vitamin A and zinc. Nutr Neurosci 2008;11:228–34. https://doi.org/10.1179/147683008X301603.
[27] Naghashpour M, Majdinasab N, Shakerinejad G, Kouchak M, Haghighizadeh MH, Jarvandi F, et al. Riboflavin supplementation to patients with multiple sclerosis does not improve disability status nor is riboflavin supplementation correlated to homocysteine. Int J Vitam Nutr Res 2013;83:281–90. https://doi.org/10.1024/0300-9831/a000170.

[28] Blaylock RL, Maroon J. Natural plant products and extracts that reduce immunoexcitotoxicity-associated neurodegeneration and promote repair within the central nervous system. Surg Neurol Int 2012;3:19. https://doi.org/10.4103/2152-7806.92935.
[29] Hamamcioglu K, Reder AT. Interferon-beta regulates cytokines and BDNF: greater effect in relapsing than in progressive multiple sclerosis. Mult Scler 2007;13:459–70. https://doi.org/10.1177/1352458506069672.
[30] Makar TK, Trisler D, Bever CT, Goolsby JE, Sura KT, Balasubramanian S, et al. Stem cell based delivery of IFN-beta reduces relapses in experimental autoimmune encephalomyelitis. J Neuroimmunol 2008;196:67–81. https://doi.org/10.1016/j.jneuroim.2008.02.014.