

The potential effects of hemp seed/evening primrose oils on the mammalian target of rapamycin complex 1 and interferon-gamma genes expression in experimental autoimmune encephalomyelitis

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Abstract

The mammalian target of rapamycin (mTOR) has a fundamental role in the metabolism, growth, and regulation of the immune system. The interferon gamma (IFN- γ) derived from T helper 1 (Th1) cells is a prominent pro-inflammatory cytokine in multiple sclerosis (MS) and its animal model, the experimental autoimmune encephalomyelitis (EAE). Due to the exclusive role of rapamycin (RAPA) in mTOR complex 1 (mTORC1) inhibition, essentially Th1 differentiation and IFN- γ production, we evaluated the potential therapeutic effects of hemp seed/evening primrose oils (HSO/EPO) in comparison with RAPA administration in EAE. To evaluate the therapeutic effects of EPO/HSO supplement in comparison with RAPA, EAE was induced using myelin oligodendrocyte glycoprotein (MOG) peptide and complete Freund's adjuvant in C57BL/6 mice. The weight, clinical score, and histological findings were evaluated. Total mRNA was extracted from local lymph nodes and qRT-PCR was used for the purpose of the genes expression level of regulatory associated protein of TORC1 (*RAPTOR*) and IFN- γ . Our results indicated that the relative expression of *RAPTOR* and *IFN- γ* genes were significantly reduced in HSO/EPO, RAPA, and RAPA + HSO/EPO treated groups in comparison with the untreated group. Interestingly, histological findings have shown that the HSO/EPO treated group remarkably regenerated the myelin sheath, but this did not occur in the case of RAPA or combined RAPA and HSO/EPO treated groups. Our findings suggest that HSO/HPO can be used as a potent immunomodulator and as a good candidate for re-myelination and downregulation of immune response for treatment of MS.

Keywords: Experimental autoimmune encephalomyelitis; Inflammation; Multiple sclerosis; Myelin sheath; Sirolimus.

INTRODUCTION

The mammalian target of rapamycin (mTOR) signaling is an important intracellular pathway in the regulation of the cell cycle and many fundamental metabolic and physiological processes. mTOR pathway forms two structurally and functionally distinct complexes identified as mTORC1 in which mTOR is bound to regulatory associated protein of TORC1 (RAPTOR), sensitive to rapamycin (RAPA) and mTORC2 in which is mTOR bound to RAPA-insensitive companion

of mTORC2 (RICTOR). mTORC2 is activated by growth factors, but, in contrast to mTORC1, seems to be nutrient-insensitive (1). The critical role for two complexes, in the activation, differentiation, and function of T helper (Th) cells has been strongly established, and in particular the expression of forkhead box P3 (FOXP3⁺), the 'master' transcription factor for T regulatory cells (Treg) (2).

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Evidence has suggested that mTORC1 tends to promote Th1 cells differentiation (3), while mTORC2 may bias the response to Th2 (4), whereas the inhibition of both mTOR complexes by RAPA is necessary for optimal Treg cell responses. Th17 cell development seems to be unrelated to TORC2 but is inhibited by RAPA in favor of Treg cells (5). Th1 cells, with the hallmark of the cytokine interferon gamma (IFN- γ) and Th17 cells interleukin-17 (IL-17) play critical roles in EAE pathogenesis, whereas Treg cells transforming growth factor- β (TGF- β) mediate immunological tolerance and reduce inflammation and prevent autoimmune diseases (6). Clinical observations suggest that defects of omega-6 and omega-3-polyunsaturated fatty acid (ω 6-PUFAs and ω 3-PUFAs) synthesis may be complicated in multiple sclerosis (MS) (7), and the risk of developing MS is related to increased dietary intake of saturated fatty acids (SFAs) (8). It has reported that, PUFAs and antioxidant deficiencies along with reduced cellular antioxidant defense mechanisms have been detected in MS patients. Also PUFAs and antioxidant treatment in EAE have reduced the clinical signs of disease (8). ω 3-PUFAs can suppress IFN- γ production in MS patients (9), and the effectiveness of ω 6/ ω 3 essential fatty acids in the body reaches the optimal level when used at the same time in the ratio of 2.5/1 (10). Accordingly, studies on the effect of HSO/EPO as a natural food/medicine with therapeutic and antioxidant properties have been performed in relapsing-remitting MS patients in clinical trials (11,12). The EPO (8-12% gamma-linolenic acid (GLA)) and HSO (6-8% GLA) are used for treating inflammatory condition. GLA is a functionally EFA that studies have confirmed its anti-inflammatory properties and it can correct the symptoms of EFA deficiency (13,14). The HSO/EPO could repair the phospholipids of cell membrane lipids by ω 6/ ω 3 PUFAs in 2.3/1 ratio and modulate immunological responses by establishing a balance between Th1 and Th2 due to antioxidant compounds (11,12). RAPA or sirolimus is the natural product that was used as an immunosuppressive therapy in organ transplantation and cancer. Unlike chemotherapy in cancer that has not been paid

to immune tendency (15), RAPA forms an immunosuppressive complex with intracellular protein, FK binding proteins (FKBP12) and blocks the activation of the mTOR kinase (16). Therefore, the current study was conducted to investigate the effects of RAPA, HSO/EPO, and the co-administration of the two agents on the weight, clinical score, histological findings, and expression of *RAPTOR* and *IFN- γ* genes in mononuclear lymph nodes cells in EAE mice.

MATERIALS AND METHODS

Animal

Female C57BL/6 mice (age, 6-8 weeks; weight, 18-22 g) were purchased from the Pasteur Institute of Iran, the Production and Research Complex. Female mice are superior to males for the study of neuropathic studies associated with MOG₃₅₋₅₅-induced EAE. MOG₃₅₋₅₅ produced behavioral signs of neuropathic hypersensitivity in females, but not males (17). All mice were housed in a specific pathogen-free environment. All experiments were performed in accordance with the animal care and the protocol of Urmia university of medical science, Urmia, I.R. Iran (Ethics committee approval No. IR.umsu.rec.1396.73).

Experimental autoimmune encephalomyelitis induction

For induction of chronic-EAE (C-EAE) in mice, the total volume of the emulsion containing a 1:1 ratio of MOG₃₅₋₅₅ (300 μ g/mouse, Sigma-Aldrich, USA)/complete Freund's adjuvant (Sigma-Aldrich, USA) was dissolved in phosphate buffer saline (PBS; Sigma-Aldrich, USA) and supplemented with *Mycobacterium tuberculosis* (500 μ g/mouse, Sigma-Aldrich, USA) and administered in two different flanking sites to each mouse. Mice received pertussis toxin (500 ng/mouse, Sigma-Aldrich, USA) i.p. at the time of immunization and 48 h later (18).

Clinical evaluation

Mice were weighed daily and assessed for EAE clinical signs. Signs of ascending paralysis of EAE were scored according to the following criteria: (0 = no clinical signs; 0.5 = partly limp tail; 1 = paralyzed tail;

2 = hind limb paresis; 2.5 = one hind limb paralyzed; 3 = both hind limbs paralyzed; 3.5 = hind legs paralyzed and weakness in forelimbs; 4 = forelimbs paralyzed and 5 = moribund or dead) (19).

Experimental animal groups

After induction of EAE in mice, on the day 15 after immunization when the clinical signs of EAE appeared, based on routine clinical scoring for EAE (19), the animals were checked and scored. EAE mice were randomly assigned to 4 groups in comparison with naive control as follows:

Group A, EAE + RAPA + HSO/EPO (n = 6): mice subjected to EAE were treated with HSO/EPO (50 μ /mouse) (11) and RAPA (1 mg/kg/50 μ) (20); group B, EAE + RAPA (n = 6): mice subjected to EAE treated with RAPA (1 mg/kg/50 μ) (20); group C, EAE + HSO/EPO (n = 6): mice subjected to EAE treated with HSO/EPO (50 μ /mouse) (11); group D, EAE control (n = 6): mice subjected to EAE treated with ethyl alcohol diluted with distilled water (1:9) (20); group E, naive control (n = 5): mice subjected to EAE treated with ethyl alcohol diluted with distilled water (1:9) (20). Mice were treated until sacrificed on day 28 after immunization. RAPA was injected daily (i.p.) into groups A and B immediately after the onset of disease symptoms and HSO/EPO was administered orally to groups A and C.

Treatment of rapamycin and hemp seed/evening primrose oils

Pure HSO and EPO were isolated from the tops of fresh varieties of industrial seeds by the cold pressing standardized method, a mechanical extraction process, in Giah Essence Agro-Industry & Phytopharm Company, Gorgan, Golestan Province, I.R. Iran. The analysis of the fatty acids of the extracted oils was determined by gas chromatography (Table 1). RAPA in powder form (Santa Cruz Biotechnology, United States) was dissolved in

1 mL of ethyl alcohol (Merck, Germany) which was diluted with distilled water. The RAPA solution was stored at 4 °C in the dark according to the manufacturer's instruction.

Histological assessment

At the end of the experiment, animals were sacrificed after administration of (i.p.) ketamine/xylazine (80/10 mg/kg) and the brain tissue was isolated to assess clinical pathology. Histologically, EAE is characterized by a predominant cerebellar or brainstem involvement. The brain of the mice in each group was enucleated and fixed in a mixture of 10% formaldehyde and deionized water for 24 h, then dehydrated in graded ethanol solutions and embedded in paraffin wax. The thickness of each section was 6-7 μ m approximately. The sections for histological examination were subjected to routine hematoxylin and eosin (H&E) and luxol fast blue (LFB) staining, to confirm infiltration of inflammatory cells, demyelination and re-myelination of axons after demyelination. We performed LFB staining that marks lipoproteins in myelin sheath and gives them a blue appearance under the light microscope (X400). LFB staining, of brain sections showed severe loss of LFB staining presenting myelin sheath loss. Demyelination and inflammation were determined by the presence of inflammatory cells and spongy tissue of the brain and the presence of strings of the non-myelin axon (21). The resulting slides each area of brain were stained with H&E and LFB was graded on a 4-point scale: 0 = not any pathology; 1 = not any tissue damage but slight inflammation; 2 = moderate inflammation, primary tissue damage and demyelination; 3 = moderate tissue destruction (demyelination, neuronal loss, tissue damage, cell death, neuronal vacuolation, neuronophagia); and 4 = necrosis (loss of all tissue elements completely with associated cellular remains). The zone with maximum tissue damage was used to evaluate each brain area (21).

Table 1. Fatty acid profiles (%) of hemp seed and evening primrose oils by gas chromatography.

| Seed oil | Palmitic acid | Stearic acid | Oleic acid | Linoleic acid | ALA | GLA | SDA | PUFA (%) |
|----------------------|---------------|--------------|------------|---------------|-----|-----|-----|----------|
| Hemp seed oil | 5.5 | 2 | 9 | 52 | 22 | 7 | 2.5 | 83.5 |
| Evening primrose oil | 5 | 1.5 | 9 | 75 | 0 | 9 | 0 | 84 |

ALA, alpha-linolenic acid; GLA, gamma-linolenic acid; SDA, stearidonic acid; PUFA, polyunsaturated fatty acids.

Table 2. Primers sequences to evaluate the expression of *RAPTOR*, *IFN- γ* , and *β -actin* genes in lymph nodes cells.

| Target gene | | Primer sequence | Product size (bp) |
|---------------------------------|---------|----------------------------|-------------------|
| <i>β-actin</i> | Forward | 5'CGTTGACATCCGTAAGACC 3' | 285 |
| | Reverse | 5'CAGTAACAGTCCGCCTAGAA 3' | |
| <i>RAPTOR</i> | Forward | 5'ACAGCCATCTACAACGGAAA 3' | 179 |
| | Reverse | 5'TATCTGACACGCTTCTCCAC 3' | |
| <i>IFN-γ</i> | Forward | 5'AACTGGCAAAAAGGATGGTGA 3' | 153 |
| | Reverse | 5'GCTGTTGCTGAAGAAGGTAG 3' | |

RAPTOR, associated protein of mammalian target of rapamycin complex 1; IFN- γ , interferon-gamma.

Quantitative real time-polymerase chain reaction

In order to investigate the expression of *IFN- γ* and *RAPTOR*, real time-polymerase chain reaction (qRT-PCR) was performed. The total RNA was extracted from the lymph node cells using kit (Gene All, South Korea) after separation from the mice and the isolated RNA was reverse transcribed using random hexamer primers and reverse transcriptase kit (Gene All, South Korea).

The extracted RNA purity was measured by measuring the ratio of optical density at 260 nm to 280 nm.

The cDNA was amplified using the SYBR-green PCR master mix kit (Ampliqon, Denmark) according to the advice of the manufacturer's directions. RT-PCR with gene-specific primers for *RAPTOR*, an essential component of mTORC1), *IFN- γ* and *β -actin* was done. RT-PCR with cDNAs of specific *RAPTOR*, *IFN- γ* , and *β -actin* were performed in the following condition: initial denaturation; 95 °C for 51 s, annealing; 60.5 °C for 60 s and *β -actin* were used as an internal control. Sequences of primers are presented in Table 2.

A melting curve analysis was used to verify the specificity of the amplification reactions. For relative quantitation, the $2^{-\Delta\Delta CT}$ formula was used (22-24).

Statistical analysis

For statistical analysis and differences between groups, one-way analysis of variance (ANOVA) was performed, and all experiments were repeated in duplicate. $P \leq 0.05$ was considered as statistically significant. Data presented as mean \pm standard error of mean (SEM).

RESULTS

Clinical assessments

Clinical assessments include weight and clinical score (CS) as described in material and methods. The body weight of HSO/EPO treated group was significantly increased in comparison with EAE, RAPA + HSO/EPO, and RAPA groups (Fig. 1). The highest scores of mice were found in the RAPA + HSO/EPO and EAE groups, respectively. The lowest score was found primarily in the HSO/EPO group. ANOVA analysis showed that differences in clinical scores were statistically significant among the five groups. Furthermore, the comparisons among the different groups showed significantly higher and lower clinical EAE scores in RAPA + HSO/EPO and HSO/EPO respectively, 28 days after induction. We observed that HSO/EPO treatment significantly reduced the development of EAE (Fig. 2).

Histological analysis

Sections were stained with H&E and LFB to evaluate the infiltration of inflammatory cells, spongiform zones, and loss in the brain tissue of EAE. To assess demyelination and re-myelination after demyelination, we used the previous described method (21,25) and showed a presence of spongiform degenerations in the brain tissue, which confirmed the demyelinated zones (21).

Histological analysis (Fig. 3) showed that treatment with HSO/EPO significantly reduced the infiltration of inflammatory cells and promoted re-myelination in comparison with RAPA, RAPA + HSO/EPO, and EAE control groups. As shown in (Fig. 3 A_{2,3} and B_{2,3}), co-administration of both RAPA and HSO/EPO was associated with more demyelination compared to RAPA and the HSO/EPO treated groups ($P = 0.000$).

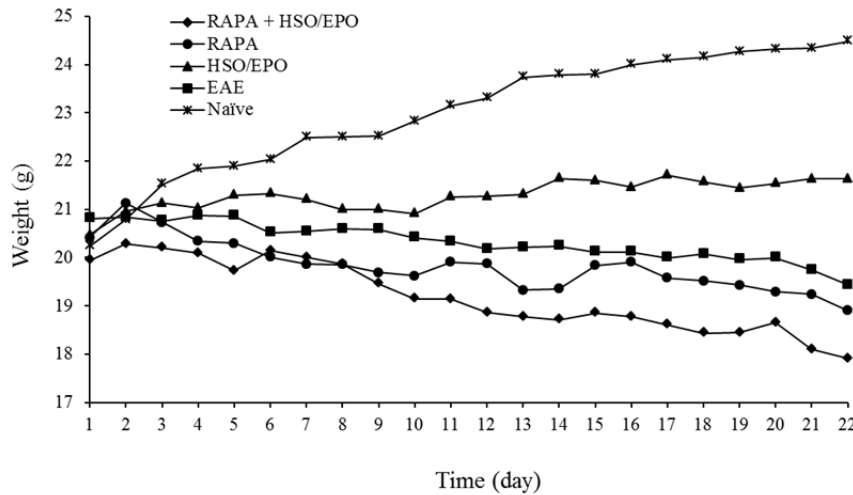


Fig. 1. Weighing of EAE animals in comparison with naïve mice. Data presented as mean \pm SEM. EAE, experimental autoimmune encephalomyelitis; RAPA, rapamycin; HSO/EPO, hemp seed oil/evening primrose oil.

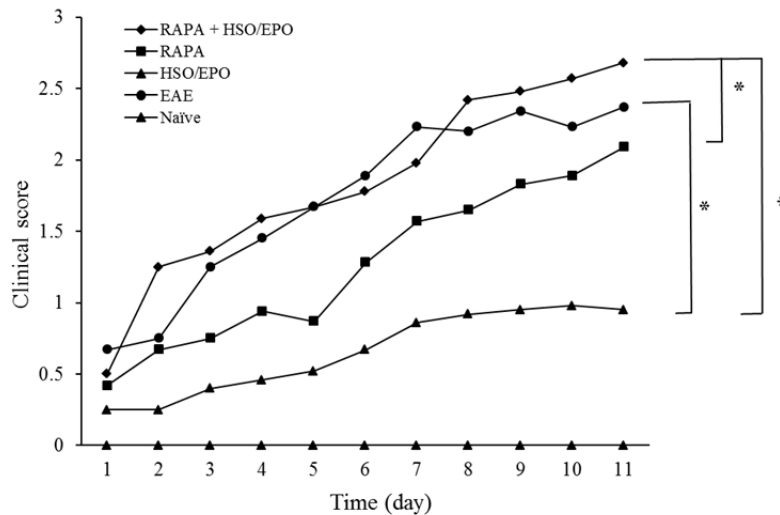


Fig. 2. Clinical course of EAE in animals. Data presented as mean \pm SEM. * P value \leq 0.05 is significant. EAE, experimental autoimmune encephalomyelitis; RAPA, rapamycin; HSO/EPO, hemp seed oil/evening primrose oil.

We found that the anti-inflammatory effects of HSO/EPO are uniquely effective than RAPA, and this effect is related to the quality of the combination of fatty acids and antioxidant properties, which prevents demyelination process and promotes regeneration of myelin sheath. In most cases of HSO/EPO group, the white matter was judged free of inflammatory cells. These results indicate that administration of HSO/EPO after EAE induction can induce specific tolerance and eliminate acute tissue damage associated with clinical EAE.

The relative expression of mTORC1 and IFN- γ genes in the extracted mononuclear cells

To evaluate the anti-inflammatory effects of HSO/EPO in comparison with rapamycin, we analyzed the expression patterns of *RAPTOR* and *IFN- γ* genes in all groups of the lymph nodes. As illustrated in (Fig. 4), it has shown that the expression of *IFN- γ* mRNA, as well as *RAPTOR*, is significantly reduced in mice treated with HSO/EPO in comparison with the EAE group (*IFN- γ* , $P = 0.003$; *RAPTOR*, $P = 0.000$) but the expression of *IFN- γ* and

mTORC1 mRNAs are not completely suppressed as we can see in rapamycin treated groups. Although the expression of *mTORC1* in HSO/EPO, rapamycin + HSO/EPO and rapamycin treated groups is significantly down-regulated, but due to the fundamental roles of mTORC1 in cell metabolism regulation, the complete down-regulation

of mTORC1 is not interested. Thus, from the point of this view, only the HSO/EPO treated group is not statistically different from normal mice. Interestingly, similar to rapamycin, HSO/EPO has a great effect on *IFN-γ* down-regulation which suggests the immunomodulatory properties of HSO/EPO ($P = 0.000$).

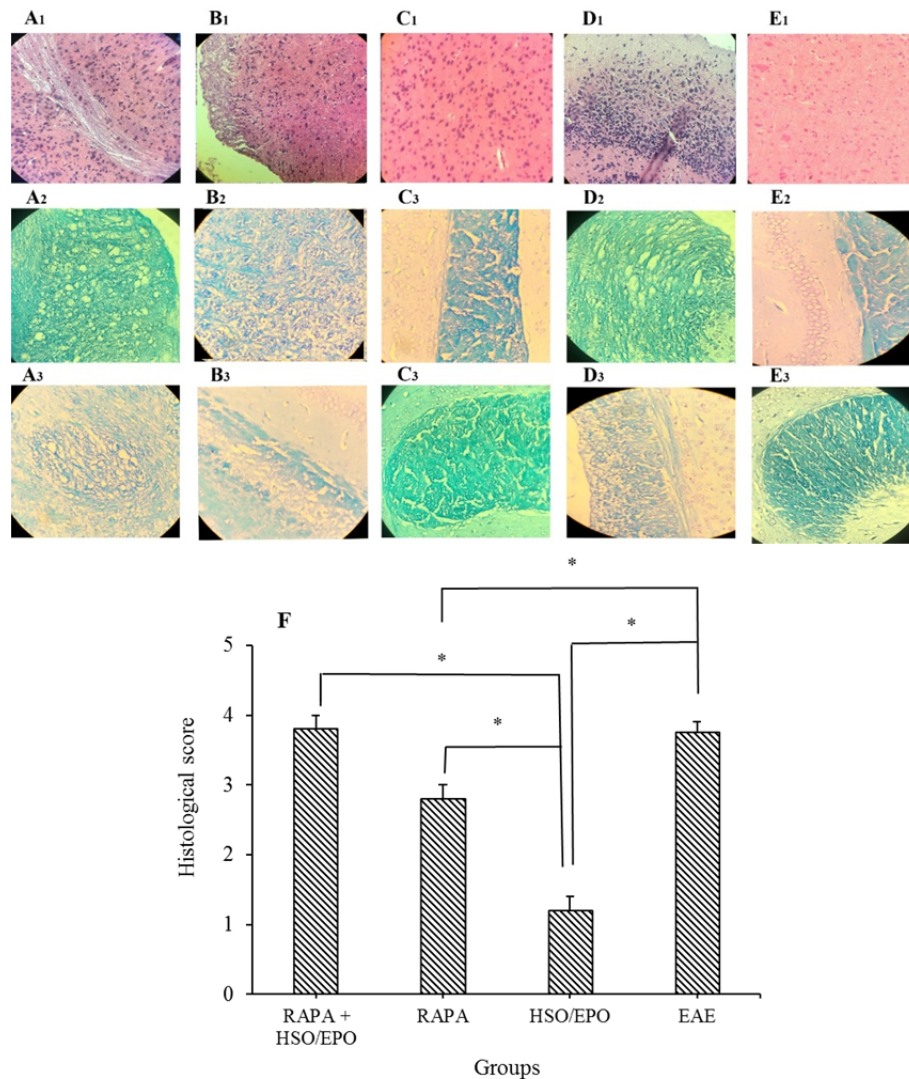


Fig. 3. Pathological analysis of brain sections. Group A, RAPA + HSO/EPO-treated mice showed (A₁) a large number of inflammatory cells, strings of the non-myelin axon, neuronophagia, (A₂) vacuolation, spongy lesions, and (A₃) extensive demyelination; group B, rapamycin-treated mice showed (B₁) infiltration of inflammatory cells, (B₂) spongiotic zones, and (B₃) demyelination; group C, HSO/EPO-treated mice showed (C₁), a few inflammatory cells and (C_{2,3}) without spongy lesions and demyelination; group D, EAE mice showed (D₁) extensive focal inflammatory cells, (D₂) extensive vacuolation, zones of spongy degeneration, and (D₃) demyelination; group E₁₋₃, the section of the brain of naive mice exhibited no clinical signs. The first row was stained with H&E, the second and third rows were stained with LFB; F, histological score: 0 = no pathology, 1 = no tissue damage but minor inflammation, 2 = modest inflammation, prime tissue damage and demyelination, 3 = moderate tissue damage (demyelination, neuronal loss, tissue damage, cell death, neuronal vacuolation, and neuronophagia), 4 = necrosis (loss of all tissue elements entirely with associated cellular remains). Data presented as mean ± SEM. * P value ≤ 0.05 is significant. EAE, experimental autoimmune encephalomyelitis; HSO/EPO, hemp seed oil/evening primrose oil; RAPA, rapamycin; H&E, hematoxylin and eosin; LFB, luxol fast blue.

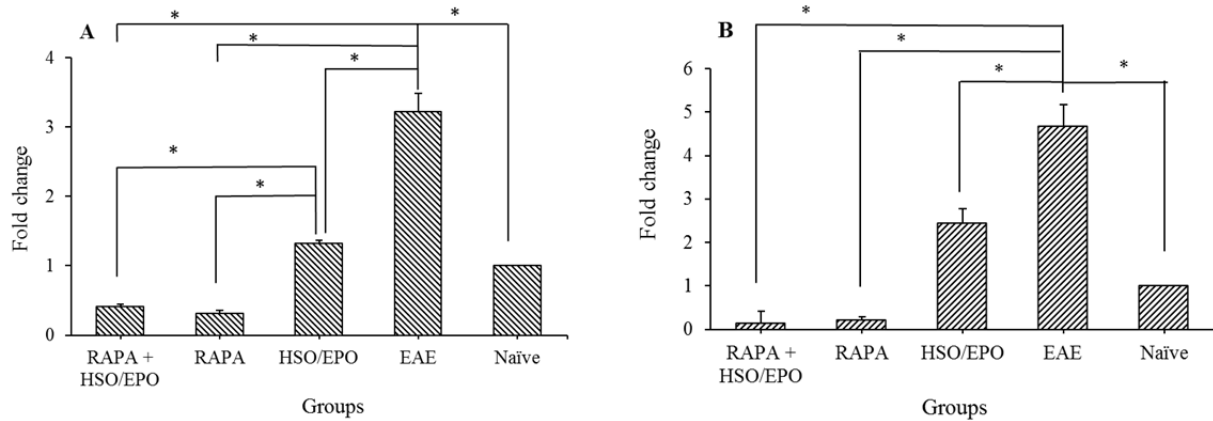


Fig. 4. The fold changes in the mRNA expression of *mTORC1* and *IFN- γ* in the lymphocytes. The expression of (A) *RAPTOR* and (B) *IFN- γ* in the HSO/EPO had a significant reduction compared with the EAE group. Data presented as mean \pm SEM. * P value \leq 0.05 is significant. mTORC1, mammalian target of rapamycin complex 1; IFN- γ , interferon gamma; RAPTOR, regulatory associated protein of TORC1; HSO/EPO, hemp seed oil/evening primrose oil; EAE, experimental autoimmune encephalomyelitis.

DISCUSSION

MS is a chronic inflammatory and neurodegenerative disease of the brain and spinal cord, which leads to disability and functional loss due to demyelination and neuronal injury (26). SFAs, animal fat without fish fat is positively correlated with MS mortality. While PUFAs may not only exert immunosuppressive actions through their incorporation in immune responses but also may affect cell function within the central nervous system (CNS). Antioxidants can support cellular defenses in various ways, including radical scavenging, interfering with gene transcription, mRNA expression, enzyme activity, and chelation. Both dietary antioxidants and PUFAs have the potential to reduce disease symptoms by targeting specific mechanisms and supporting recovery in MS (8). HSO has been used to treat various disorders for many years in traditional medicine. Recent clinical trials have known HSO as a functional food (27). HSO has over 80% PUFAs and is a remarkably rich source of the two essential fatty acids (LA and α -LA). The ω 6/ ω 3-PUFA ratio in HSO is routinely between 2:1 and 3:1, which is considered optimal for human health (10). From a nutritional point of view, up to 7% GLA and 2.5% stearidonic acid (SDA or STA) is very remarkable (28). The corporation of both GLA and SDA in HSO, naturally at a

favorable ω 6/ ω 3 ratio of 2:1, allows this enzymatic phase with delta-6-desaturase to be efficiently bypassed (29). The delta-6-desaturase, which is the slowest and rate-limiting step in the metabolic pathway to GLA, catalyzes this reaction. GLA is rapidly elongated to dihomo- γ -LA (DGLA) by the elongase enzyme, and DGLA is acetylated and incorporated into the cell membrane phospholipids. DGLA competes with the arachidonic acid for cyclooxygenase (COX) enzymes, and the metabolites of DGLA and arachidonic acid that are produced by the prostaglandins E3 and E2, respectively have actions that oppose each other. Following an inflammatory stimulus in MS-like diseases, proinflammatory eicosanoids derived from arachidonic acid are prostaglandin E2, whereas GLA and DGLA produce anti-inflammatory eicosanoids including prostaglandin E1 and prostaglandin E3 that inhibit IFN- γ , tumor necrosis factor- α , IL-1 β and IL-6 (30). Analysis revealed that HSO contains over 3.6-6.7 g antioxidant/kg oil including terpenes, phytosterols, and tocopherols and polyphenols (28). The antioxidant properties of HSO are scavenger free radicals and regulate signaling pathways to modulate inflammatory reactions (31,32). EPO is rich in GLA that precursors of anti-inflammatory eicosanoids, which are elements of cell membranes. The metabolism of dietary GLA ultimately leads to PGE1, which has an effective anti-inflammatory

activity and is regularly recommended for the treatment of inflammatory and autoimmune disorders (33). The earliest results in the use of EPO and colchicine combined therapy in MS patients suggest that it may be of considerable value (34). Rapamycin is a natural product produced by *Streptomyces hygroscopic* strain, which was used as an immunosuppressive therapy in organ transplantation and certain types of cancer (16). The adverse side effects of RAPA include anemia, thrombocytopenia, nausea, headache, fever, urinary tract infection, and interstitial pneumonitis (35), limited the usefulness of this drug. RAPA predominantly inhibits differentiation of effector T cells by inhibiting mTORC1 and mitigating Th1 and Th17 differentiation but not Th2 cells (36). As shown in our experiment, the expression of mTORC1 mRNA levels was elevated in EAE mice while, an immunomodulatory agent (HSO/HPO) could reduce the inflammatory condition and improve re-myelination in comparison with RAPA in EAE mice alone, but not along with RAPA. In our previous study, HSO/EPO was shown to reduce the secretion of inflammatory cytokines. The mechanism by HSO/EPO exerts its beneficial effects is not limited to attenuating the inflammation, but it is also related to its effects on phospholipids synthesis in erythrocytes membrane cells in MS patients (37).

Analyzes have shown that the expression of *mTORC1* and *IFN- γ* mRNA was significantly reduced both in RAPA and HSO/EPO treated groups compared to EAE group. According to previous findings (38), our data indicated that completely down-regulation of *RAPTOR* is not appropriate and it is largely because of the contribution of *RAPTOR* in Th2 cell differentiation. Also, histological findings showed that HSO/EPO group had less activated inflammatory cells and spongy degeneration in the brain tissue. Likely, HSO/EPO affects the activation of inflammatory cells and promoting re-myelination in the CNS tissue. Since re-myelination occurs during the initial phases of the disease, this is rare in more developed stages (39). Additionally, we found that co-administration of RAPA and HSO/EPO

was not effective as HSO/EPO, mainly due to the deleterious effects of RAPA on the suppression of the mTORC1 pathway and consequently as a result of the accumulation of fatty acids in the blood stream and causes a condition similar to the metabolic syndrome. Evidence suggests that chronic inhibition of mTOR with RAPA leads to an exacerbation of hyperglycemia and insulin resistance (40). Since the cells cannot absorb fatty acids from the bloodstream, the accumulation of fatty acids causes metabolic disturbances to exacerbate inflammation and thus lead to myelin degradation. Therefore, that treatment with RAPA+ HSO/EPO led to greater vacuolation, spongy lesions, and demyelination in the brain. The addition of RAPA to the HSO/EPO re-myelination model appeared to prevent re-myelination from occurring during the treatment period. The findings with the identified adverse effects of RAPA on the uptake of fatty acids in the RAPA+ HSO/EPO group may well explain the reduction in weight.

Inhibitory effects of RAPA on mTOR not only enhance demyelination in the HSO/EPO model but also provide insight into signaling mechanisms through mTOR that regulate re-myelination. Therefore, our finding according to reports of others (41) indicating the efficacy of RAPA-mediated inhibition of mTOR, possibly reduced signaling through *RAPTOR*, as well as spongy degeneration and demyelination, was observed in mice treated with RAPA alone and RAPA+ HSO/EPO. These data support the role of mTOR, and more specifically mTORC1, as a positive mediator in the context of re-myelination. In the RAPA treated group, despite the IFN- γ suppression, there is a significant demyelination and infiltration of inflammatory cells. The RAPA inhibitory effects on mTORC1 expression cause disturbance in the USFAs in cellular structures and USFAs cannot be incorporate in cell membranes, especially myelin (42). Also RAPA or mTORC1 inhibitor promotes lipolysis by enhancing protein kinase A-mediated phosphorylation of the hormone-sensitive lipase (43). It was reported that ω 3-LC-PUFAs mediated function modulates mTORC1 activity and may increase

Treg cell count and function (44). Therefore, the quality of fatty acids, including reduced proportions of $\omega 6/\omega 3$ fatty acids or intake increased $\omega 3$ -PUFAs, could alleviate autoimmune diseases by modulating Th1/Th2/Th17 cells and upregulating Treg cells through control of mTOR signaling such as RAPA (37,45,46). Finally, HSO/EPO treatment improved the histological EAE score by attenuating infiltration of inflammatory cells and promoting re-myelination, suggesting a protective effect in CNS tissues. The route of administration, oral, is another beneficial effect of our experiment. It is suggested that oral administration of polyphenolic compounds in HSO/EPO, which contains a mixture of various chemicals with potential bio-reactivity (37), could be a reasonable way of using this supplement in MS patients.

CONCLUSION

Taken together, our research has shown that HSO/EPO may be an effective treatment for cognitive impairment of MS patients to promote re-myelination, whereas RAPA treatment has not been reported to be effective in EAE or MS treatment by promoting myelin sheath regeneration.

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