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Original Article

Hemp seed/evening primrose oil affects expression of STAT3, IL-17, and FOXP3$^+$ in experimental autoimmune encephalomyelitis

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Abstract

T helper (Th)-17 mediate inflammation in both peripheral tissues and the central nervous system. Signal transducer and activator of transcription factor3 (STAT3) is required for Th-cell pathogenicity and its activation in the brain has been demonstrated during the acute phase of experimental autoimmune encephalomyelitis (EAE) through the mammalian target of rapamycin (mTOR) signaling. Rapamycin (RAPA), an inhibitor of mTOR, can drive Forkhead box P3 (FOXP3$^+$) induction as a regulatory factor. The aim of this study was to determine the effects of hemp seed/evening primrose oils (HSO/EPO) supplement on the expression of FOXP3$^+$, STAT3, and interleukin (IL)-17 genes in EAE lymph nodes. EAE was induced by myelin oligodendrocyte glycoprotein peptide in mice, and then the mice were assigned to three treatment groups compared to two control groups (EAE and naive). The histological findings of the spinal cord were evaluated. To determine the expression of FOXP3$^+$, STAT3, and IL-17 genes in the lymphocytes, qRT-PCR was used. Our results showed that EAE severity was reduced in HSO/EPO mice by reducing the expression of STAT3 and IL-17 genes and increasing the expression of FOXP3$^+$ gene, which was confirmed by slight inflammation in the spinal cord. Histological findings showed a significant improvement in the HSO/EPO group. Our findings suggest that the HSO/EPO treatment can be used to ameliorate the demyelination of spinal cord, which was confirmed by immunological and histological findings.

Keywords: Autoimmune; Encephalomyelitis; Lymphocytes; Rapamycin; T cell.

INTRODUCTION

Multiple sclerosis (MS), an inflammatory disease and neurological disability, is associated with upregulation in T helper (Th)-1/Th-17 cells and acceleration of the autoimmune disease. In contrast, the conversion of a Th-1/Th-17 profile to the development of T regulatory cells (Treg) or the inhibition of Th-17 deviation is beneficial for the repression immune responses and modifying the inflammation (1). MS patients have abnormalities in Forkhead box P3 (FOXP3$^+$) known as a master regulator in the development and function of regulatory T cells (Treg), which are involved in maintaining peripheral tolerance and control of immune responses by introducing suppressive effects on activated immune cells (2). The mammalian target of rapamycin (RAPA, mTOR) signaling, an essential regulator, regulates Th cell differentiation through selective activation of two complexes; mTOR complex 1 (mTORC1) and mTORC2 signaling. mTORC1 signaling control Th1 and Th17 differentiation, while mTORC2 signaling regulates Th2 cells (3). Damaged mTOR do not lead to differentiation of Cluster of differentiation 4 (CD4+) T cells into effector cells under suitable bias conditions but activate the of FOXP3$^+$ regulatory cells (4).

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This failure to differentiate into the effector T cells in the defect of mTOR was related to reducing the stimulation of the signal transducer and activator of transcription factor3 (STAT3) signaling that controls T cell differentiation, multiplication, and survival. Inhibition of the mTOR-STAT3 pathway is associated with suppression of Th-17 cell differentiation (5). Thus, the production of the cytokine of interleukin (IL)-17 depends on the activation of STAT3 (4). RAPA, an inhibitor of mTOR, induces $FOXP3^+$ by directly acting on $CD4^+$ T cells (6), and ameliorates experimental autoimmune encephalomyelitis (EAE) by suppressing the mTORC1-STAT3 pathway via promoting immunosuppression (7). On the other hand, several studies have reported that abnormalities of polyunsaturated fatty acid (ω3-PUFAs, ω6-PUFAs) synthesis may be involved in MS pathogenesis (8). PUFAs can modulate many of the signal transduction mechanisms operating in neuronal membranes (9).

Dietary PUFAs such as hemp seed/evening primrose oils (HSO/EPO) supplement can affect inflammatory functions and cytokines production in favor of MS patients (10). The HSO has a high amount of essential fatty acids (EFAs), typically at a favorable ω6-linoleic acid (LA)/ω3-alpha-linolenic acid (ALA) for human membrane (11) such as myelin. Phenolic compounds not only exhibit potent antioxidative properties but may also act on specific signaling pathways for regulation of inflammatory responses (12). The EPO content of gamma-linolenic acid (GLA) is often recommended for the inflammatory and autoimmune conditions for MS patients (13). In our previous work the ameliorate effects of HSO and EPO on EAE have been proven (14). Therefore, our rationale was to investigate the effects of HSO/EPO supplement in comparison with RAPA on the expression of IL-17, STAT3, and $FOXP3^+$ genes of lymphocytes and histological assessments on the spinal cord in EAE course.

MATERIALS AND METHODS

Materials

Myelin - oligodendrocyte glycoprotein $(MOG)_{35-55}$ peptide, Phosphate buffer saline (PBS), Pertussis toxin and complete Freund's adjuvant (CFA) were purchased from Sigma, (St. Louis, USA). RAPA was purchased from Santa Cruz Biotechnology (Texas, USA). Ethyl alcohol and formaldehyde were procured from Merck (Darmstadt, Germany). RNeasy and superscript reverse transcriptase kits were purchased from Gene All (Seoul, South Korea). Power SYBR Green real time polymerase chain reaction (RT-PCR) master mix kit was supplied by Ampliqon (Stenhuggervej, Denmark).

Animal

Female C57BL/6 mice (aging 6-8 weeks) were obtained from the Pasteur Institute of Iran, the Production and Research Complex, (Teharn, I.R. Iran). All animals were kept in pathogen-free environment. All experimental manipulations were approved by the Animal Research Ethics Committee of Urmia University of Medical Science, Urmia, I.R. Iran (Ethics committee approval No. IR.umsu.rec.1396.73) and performed in accordance with National Institute of Health Guide for the Care and Use of Laboratory Animals.

Experimental autoimmune encephalomyelitis induction

Mice were immunized for chronic progressive disease in day 0 in flanks subcutaneously with MOG_{35-55} peptide (300 μg/mouse) dissolved in 100 μL of PBS emulsified in an equal volume of CFA containing heat-killed *Mycobacterium tuberculosis* (500 μg/mouse, Sigma-Aldrich, USA). Mice received two injections intraperitoneally (i.p.) of pertussis toxin (500 ng/mouse, Sigma-Aldrich, USA) dissolved in 100 μL of PBS at the time of immunization and 48 h later. Mice were scored for clinical signs of disease already published (14)

Experimental animal groups

Thirty mice were used to perform the experiments. Eighteen EAE mice were randomly assigned to three groups (EAE/administered) and twelve mice were used as control groups (EAE and naive). Six mice per each group were used to conduct the experiments:

Group A, EAE mice treated with RAPA (1 mg/kg; i.p.) (15) and HSO/EPO (50 µL/mouse) P.O. (16); group B, EAE mice treated with RAPA (1 mg/kg/50 µL; i.p.); group C, EAE mice received HSO/EPO (50 µL/mouse) P.O.; group D, EAE mice treated with 1% ethyl alcohol diluted with distilled water. i.p. (15); group E, naive mice treated with 1% ethyl alcohol diluted with distilled water .i.p. When the clinical signs of EAE began to appear and the mice showed the beginning of the active disease, they were treated and treatment continued until they were killed. RAPA was injected daily into groups A and B mice immediately after the onset of disease signs (about 14 days after immunization) and HSO/EPO was administered P.O. to groups A and C.

Preparation of rapamycin and hemp seed/evening primrose oils

Pure HSO and EPO were isolated from commercial seeds in the standard cold-pressed method at Giah Essence Agro-Industry & Phytopharm Company, Gorgan, Golestan Province, I.R. Iran. RAPA powder was dissolved in 1 mL ethyl alcohol and then, diluted with distilled water. The RAPA solution was stored at 4 °C in the dark according to the manufacturer's instruction. The control solution included only 1% ethyl alcohol and diluted with distilled water.

Histological assessment of spinal cords

At the end of the EAE study (Fig. 1), the vertebral columns of the mice in each group were enucleated and fixed with 10% formaldehyde and deionized water solution for 24 h. Then, the entire vertebral columns were carefully separated and incubated overnight at 4°C for tissue post-fixation. For histological examination, the spinal cords were decalcified for 48 h (37 °C), and the samples were washed for 12 h to eliminate decalcification solution. Spinal cords were dehydrated in ethanol solutions and fixed in paraffin wax. The fixed tissues were cut into 6-µm thick sections and prepared for routine staining of hematoxylin and eosin (H&E) for infiltration of inflammatory cells, and luxol fast blue (LFB) for demyelination and acute axonal damage monitoring.

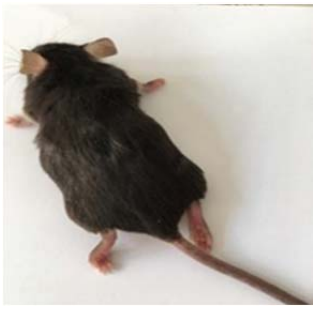

Fig. 1. Photograph of a C57BL/6 mouse with paralyzed hind limbs and tail (score of 3) following induction of experimental autoimmune encephalomyelitis.

Inflammatory lesions and damaged myelin were examined under light microscopy (×400) (17). The resulting slides in each area of the spinal cord were graded in a 4-point scale: 0 = no pathologic presentation; 1 = no 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 spinal cord area (17).

Real time polymerase chain reaction

In order to determine the expression of immunological-related genes, total RNA was extracted from lymphocytes with the RNeasy kit according to the manufacturer's instruction. The extracted RNA purity was evaluated by measuring the ratio of optical density at 260 to 280 nm. Also, RNA integrity for each gene was assessed by agarose gel electrophoresis.

Complementary DNA (cDNA) was synthesized with a Superscript reverse transcriptase. The arranged cDNAs were analyzed in duplicate by RT-PCR amplification using the Power SYBR Green PCR master mix kit. A negative control (without cDNA) was used to investigate the unwanted contamination and primer dimer formation. RT-PCR with cDNAs of specific IL-17, STAT3, FOXP3^{+}, and β-actin2 was performed at 40 cycles of denaturation for 15 s at 95 °C, annealing for 60 s at 60.5 °C. RNA integrity used for RT-PCR was confirmed by β-actin2 synthesis as a positive control reaction. Sequences of primers presented in Table 1.

Table 1. Primers sequences to evaluate the expression of IL-17, STAT3, FOXP3$^+$, and β-actin2 genes in lymph nodes cells.

Target gene		Primer sequence	Product size (bp)
β-actin2	Forward	5′-CGTTGACATCCGTAAAGACC-3′	285
	Reverse	5′-CAGTAACAGTCCGCCTAGAA-3′	
FOXP3$^+$	Forward	5′-CTGTGCCTGGTATATGCTCC-3′	133
	Reverse	5′-TAGGGTTGGGCATTGGGTT-3′	
STAT3	Forward	5′-CTCGGGGTTGGTTGTTAGA-3′	223
	Reverse	5′-ATGGAAAGGCTATGCTGTGT-3′	
IL-17	Forward	5'-TCTGTGTCTCTGATGCTGTTG-3′	238
	Reverse	5'-TATCAGGGTCTTCATTGCGG-3'	

FOXP3$^+$, Forkhead box P3; STAT3, Signal transducer and activator of transcription factor3; IL-17, Interleukin-17.

A melting curve analysis was used to confirm the specificity of the amplification reactions. For a relative quantification calculation, the $2^{-\Delta\Delta CT}$ formula was used (18).

Statistical analysis

Data were analyzed using statistical packages for the social sciences (SPSS version 16.0) software, and are presented as the mean ± standard error of the mean (SEM). For statistical analysis and differences between groups, one-way analysis of variance (ANOVA) was performed and followed by Tukey post hoc test. RT-PCR results of all experiments were repeated in duplicate. $A\ P \leq 0.05$ was considered statistically significant.

RESULTS

Composition of the fatty acids in hemp seed/evening primrose oils

The analysis of the fatty acids of the extracted oils was determined by gas chromatography (Table 2). LA and ALA levels were very high in ratio 2:1, as well as GLA and stearidonic acid. Because these two groups of EFAs perform distinct and complementary functions in membrane cells such as myelin, this ratio needs to be achieved from dietary intake.

Histological analysis

Histological evaluations were performed on paraffin-embedded sections of the spinal cords samples after the 28th day. Sections were stained with H&E and LFB to evaluate cells infiltration, demyelination, axonal loss, and spongiform changes (17).

Pathological examinations of RAPA + HSO/EPO, RAPA, and EAE control groups in spinal cords in comparison with those of HSO/EPO group revealed extensive demyelination in the white matter areas associated with immune cell infiltrations (Fig. 2). In previous studies (14), histological analysis of brain sections has showed that treatment with HSO/EPO significantly reduced infiltration and demyelination in comparison with RAPA, RAPA + HSO/EPO, and EAE control groups due to inhibition of mTORC1 activity by RAPA. RAPA inhibited the remyelination of central nervous system (CNS) mice and accelerated demyelination.

The relative expression of IL-17, STAT3, and FOXP3$^+$ genes in the extracted mononuclear cells

To confirm the accuracy of the extraction of RNA, the RNA integrity of genes including interleukin (IL)-17, STAT3, FOXP3$^+$, and β-actin2 was evaluated by agarose gel electrophoresis with ladder 50 bp (Fig. 3).

The treatment of RAPA, HSO/EPO, or combination of both agents induced a significant elevation in the expression of FOXP3$^+$ and reduction in the IL-17 and STAT3 genes in HSO/EPO, RAPA, and RAPA + HSO/EPO mice compared to EAE control mice. Although this increase in FOXP3$^+$ and reduction of STAT3 and IL-17 expresion in RAPA groups was more severe than the HSO/EPO group, no expected recovery was observed, especially in the RAPA + HSO/EPO group. The statistically significant differences were found only among the five groups of mice, as illustrated in Fig. 4. Also, the parameters showed that the results in the HSO/EPO group were closer to the naïve healthy group and there was no significant difference between the HSO/EPO supplement and the healthy group.

Table 2. Fatty acid composition (%) of HSO and EPO analyzed using gas chromatography.

Seed oil	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	ALA	GLA	SDA	%PUFA
HSO	5.5	2	9	52	22	7	2.5	83.5
EPO	5	1.5	9	75	0	9	0	84

HSO, Hemp seed oil; EPO, evening primrose oil; ALA, alpha-linolenic acid; GLA, gamma-linolenic acid; SDA, stearidonic acid; PUFA, polyunsaturated fatty acids (ω6/ω3-PUFAs).

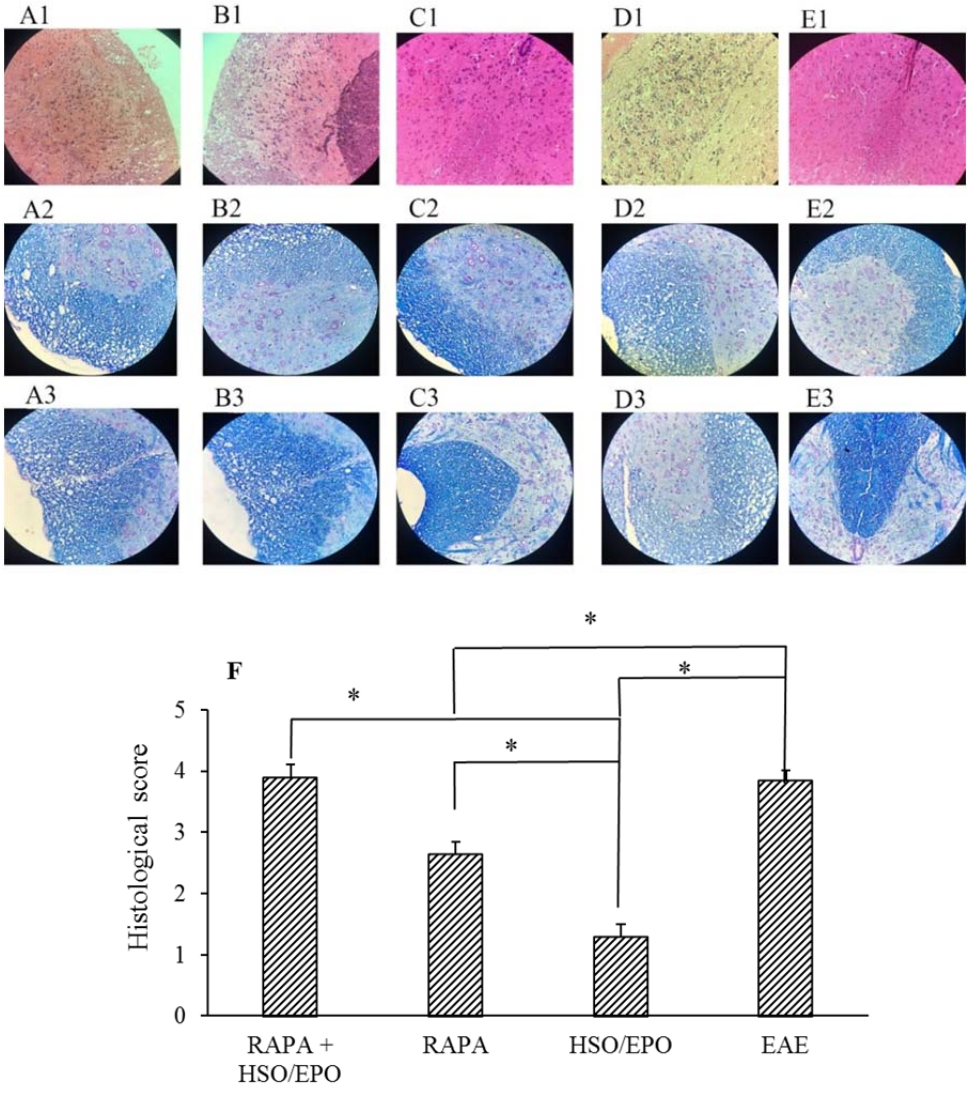

Fig. 2. Pathological analysis of the spinal cord sections. Group A, RAPA + HSO/EPO-treated mice showed (A_1) infiltration of inflammatory cells, ($A_{2,3}$) vacuolation, spongy lesions, and extensive demyelination; Group B, rapamycin-treated mice showed (B_1) numerous inflammatory cells, ($B_{2,3}$) spongiotic zones and demyelination; extensive demyelination; Group C, HSO/EPO-treated mice showed (C_1), a few inflammatory cells and ($C_{2,3}$) without spongy lesions and demyelination; Group D, EAE control mice showed (D_1) severe infiltration of numerous immune cells and, ($D_{2,3}$) extensive vacuolation, zones of spongy degeneration, and demyelination; Group E, in the section of $E_{1\text{-}3}$ from naive mice exhibiting 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 pathologic presentation, 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 are presented as mean ± SEM. *Indicates significant differences between corresponding groups ($P \leq 0.05$). EAE, experimental autoimmune encephalomyelitis; HSO/EPO, hemp seed oil/evening primrose oil; RAPA, rapamycin; H&E, hematoxylin and eosin; LFB, luxol fast blue.

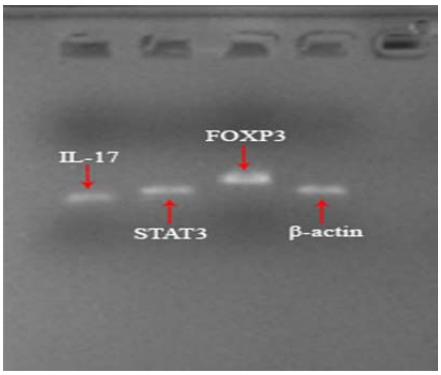

Fig. 3. The RNA integrity of genes including interleukin (IL)-17, signal transducer and activator of transcription factor3 (STAT3), Forkhead box P3 (FOXP3$^+$), and β-actin2 evaluated by agarose gel electrophoresis with ladder 50 bp (left to right).

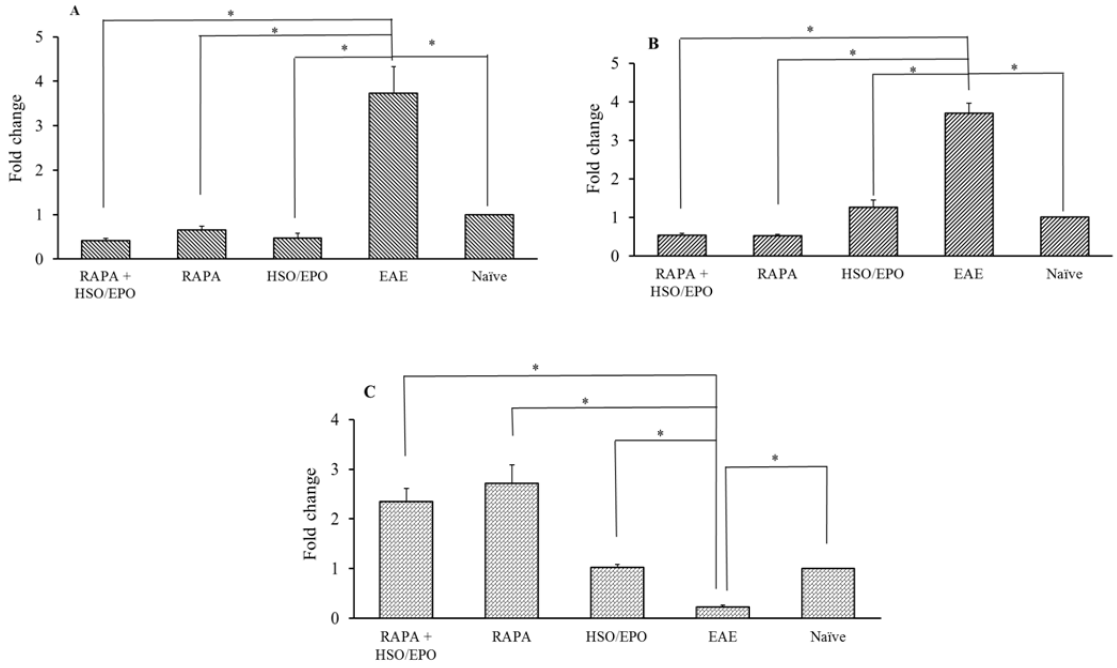

Fig. 4. The fold changes in the mRNA expression of IL-17, STAT-3, and FOXP3$^+$ genes in the lymphocytes. The expression of (A) IL-17 and (B) STAT-3 indicate the expression of IL-17 and STAT-3 in the HSO/EPO and both of RAPA groups exhibited a significant reduction compared to EAE mice. The expression of (C) FOXP3$^+$ in the HSO/EPO and both of RAPA groups indicated a significant increase compared to EAE mice. Data are presented as mean ± SEM. *Indicates significant differences between corresponding groups ($P \leq 0.05$). IL-17, Interleukin-17; STAT3, signal transducer and activator of transcription factor3; FOXP3$^+$, Forkhead box P3; HSO/EPO, hemp seed oil/evening primrose oil; EAE, experimental autoimmune encephalomyelitis.

DISCUSSION

MS is an inflammatory disease and neurologic disability of the CNS, which is associated with the destruction of the myelin sheath (1). The pro-inflammatory cytokines are produced by several types of immune T cells: Th1 cells express IFN-γ; Th2 cells express IL-4; Th17 cells express IL-17; Treg cells express FOXP3$^+$, which ameliorates other T-cells responses (19). Several studies have reconsidered the relative significance of Th1 and Th17 cells in inflammatory demyelination in MS patients and EAE (1) and confirmed a critical role for Th17 cells and expression of IL-17 in mononuclear cells (MNCs) in disease pathogenesis (20). In the CNS, phospholipase A2 (PLA2) controls the metabolism of PUFA and releases free fatty acids and lysophospholipids (Lyso-PLs) from myelin

membranes. Increased activities of PLA2 and generation of lipid mediators play a central role in oxidative stress and neuroinflammation, which is associated with neurological disorders in MS and EAE (21). The treatment with a PLA2 inhibitor results in the promotion of $FOXP3^+$, which plays a critical role in preserving immune homeostasis and preventing autoimmune diseases (22). Another study showed that PLA2 block led to the reduction of Th1 and Th17-type cytokines, as well as significant reductions in IFN-γ, TNF-α, and IL-17 concentrations (23). Also, it has been confirmed that the PLA2 pathway is essential in STAT3 phosphorylation and STAT3 are located downstream of PLA2 (24). HSO/EPO contains over 80% of the ω6/ω3 PUFAs in 2.3:1 ratio (Table 2), which is optimal for repair of myelin membranes. In the previous study, the treatment of MS patients by HSO/EPO resulted in a reduction in PLA2 (10). In the present RT-PCR results, a reduction in expression of STAT3 and IL-17, as well as an increase in $FOXP3^+$ in lymphocytes of HSO/EPO-treated mice, was observed (Fig. 4).

STATs proteins are located in the cell cytoplasm in the resting state. After phosphorylation, they migrate into the nucleus where they control the expression of cytokines gene (25). STAT3 is the downstream target molecule of mTOR and phosphorylation of both mTOR and STAT3 indicates activation of the mTOR-STAT3 pathway (7). A reduction in the expression of STAT3 by HSO/EPO is an important finding in our study since STAT3 activation was demonstrated during the acute phase of EAE and MS patients. According to our results, the loss of STAT3, the master regulators of Th17 cells, mitigates the development of EAE (26).

On the other hand, the dysbalanced production of ω6 to ω3 PUFAs can be linked to inflammation (27). Following the inflammatory stimuli, PLA2 stimulates the release of arachidonic acid (AA), and the generation of proinflammatory eicosanoids such as prostaglandin (PG) E2. Also, the imbalance of dietary intake in ω6 and ω3 PUFAs accelerates the destruction of cell membranes and displaces AA from the cell membrane by PLA2 in favor of production of PGE2 (28). Therefore, dietary intake in ω6 to ω3 PUFAs in the ratio of 2.3: 1 is essential because two groups of EFAs perform distinct and complementary functions and it has been recommended that ω6 and ω3 EFAs be given together (29). The combination of HSO and EPO oils as a dietary supplement has a potential to reduce prostaglandin E2 (PGE2) and pro-inflammatory cytokines Th1 and Th17 (16). HSO has used as a food /medicine or functional food to treat various disorders for thousands of years in traditional medicine (11). HSO is a rich source of several antioxidants including terpenes, phytosterols, tocopherols, and polyphenols with the properties of scavenger free radicals and regulation of signaling pathways to modulation inflammatory responses (12). The use of EPO and colchicine combined therapy in MS patients was suggested to be of considerable value. EPO, rich in GLA (Table 2), is a precursor of anti-inflammatory eicosanoids PGE1. PGE1 has an effective anti-inflammatory activity and is commonly recommended for the treatment of inflammatory and autoimmune disorders (13).

Under the inhibitory effects of RAPA on mTOR activity, cytokines including IL-17 and IFN-γ fail to promote upregulation, partly due to a decrease in STAT activation (4) and promotion in $FOXP3^+$ induction (6). RAPA or sirolimus, an oral macrocyclic immunosuppressive drug, displaying antitumor activities used to prevent rejection in human organ transplantation (6). RAPA ameliorates EAE by suppressing the mTOR-STAT3 pathway (7) but does not affect the expression of PLA2 or the destruction of cell membranes such as myelin (30). Regarding RAPA inhibitory effects on mTOR, in RAPA groups, the activation of STAT3 and the production of the Th17 cytokines, is not upregulated (Fig. 4). Inhibition of the mTOR pathway and lowering of phosphorylation of STAT3 are associated with an increase in $FOXP3^+$, a transcription factor promoting Treg cells versus Th1/Th17 cells (7), which was observed in RAPA groups (Fig. 4).

Our RT-PCR results indicated that HSO/EPO-treated mice did not show

significant differences with the naive healthy group. Also, the increased expression of $FOXP3^+$ in the lymph nodes of HSO/EPO-treated mice suggests that treatment induces the development of Treg cells. While, in RAPA + HSO/EPO group, although the mTOR-STAT3 pathway and expression of the IL-17 gene were completely suppressed and $FOXP3^+$ increased, we observed no improvement compared to the HSO/EPO group (Fig. 4). In contrast, treatment with RAPA alone suppressed the promotion of EAE. Low amelioration was observed due to promotion of the differentiation of Treg cells and inhibiting the generation of Th1 and Th17 cells, without significant histological improvement due to mTOR impairment (Fig. 4). Collectively, these results suggest that the adverse effects of RAPA on the metabolism and the uptake of fatty acids in the RAPA + HSO/EPO group can affect weight reduction in mice. This can be considered in two respects. Firstly, RAPA cannot inhibit the destructive effects of PLA2 (30) and promotes lipolysis by enhancing protein kinase A-mediated phosphorylation of the hormone-sensitive lipase (31). Secondly, mTORC1 inhibition by RAPA treatment leads to downregulation of a downstream effector of mTORC1, sterol regulatory element-binding protein, a lipogenic transcription factor that regulates the transcription of fatty acid synthesis enzymes (32). Any post-demyelination treatment should strengthen remyelination and help repair neuron membranes, while RAPA inhibits active myelination by inhibiting the Akt/mTOR signaling pathway (33). In long-term treatments with RAPA, demyelination becomes more severe and the condition of the organ becomes worse. Histological findings of the HSO/EPO-treated mice showed less inflammation and no demyelination in the spinal cord, while RAPA + HSO/EPO-treated mice had the highest rate of inflammation and demyelination as same as EAE control mice (Fig. 2). Results showed that administration of RAPA + HSO/EPO was not as effective as HSO/EPO. The suppression of the mTORC1 pathway by RAPA results in the accumulation of fatty acids in the blood stream, causing metabolic syndrome and promoting inflammation. It has been shown that chronic inhibition of mTOR by RAPA leads to an exacerbation of hyperglycemia and insulin resistance (34). Since body cells cannot uptake fatty acids from the bloodstream, the accumulation of fatty acids causes metabolic disorders to exacerbate inflammation and thus lead to myelin degradation. Therefore, the HSO/EPO + RAPA seems to prevent remyelination due to the adverse effects of RAPA on the metabolism of fatty acids.

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