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The Effects of Evening Primrose/Hemp Seed Oil Compared to Rapamycin on the Gene Expression of Immunological Parameters in Experimental Autoimmune Encephalomyelitis Splenocytes

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ABSTRACT

Mouse model of multiple sclerosis (MS) is used for the inflammatory demyelinating disease. Rapamycin (RAPA) may contribute to the reduction of inflammatory responses to experimental autoimmune encephalomyelitis (EAE). Due to its adverse side effects, identifying new therapeutic agents is important. We investigated the transcriptional effects of evening primrose/hemp seed oil (EP/HS oil) compared to RAPA on the expression of immunological factors genes in spleen cells of EAE mouse models.

We firstly induced EAE mice by injection of myelin oligodendrocyte glycoprotein (MOG). Then, the EAE mice treated and untreated with EP/HS oil were evaluated and compared with naïve mice. The spinal cords were examined histologically. The immunological factors including genes expression of the regulatory-associated protein of mammalian target of rapamycin (RAPTOR), regulatory-associated companion of mammalian target of rapamycin (RICTOR), interferon (IFN)-γ, interleukin (IL)-10, signal transducer and activator of transcription factors (STAT3), forkhead box P3 (FOXP3), and IL-17 of splenocytes were evaluated by real time-polymerase chain reaction (RT-PCR).

The data showed that EP/HS oil was able to reduce the severity of EAE and inhibited the development of the disease. EP/HS oil treatment significantly inhibited the expression of RAPTOR, IFN-γ, IL-17, and STAT3 genes and promoted the expression of RICTOR, IL-10, and FOXP3 genes.

In conclusion, the EP/HS oil is likely to be involved in transcription of factors in favor of EAE improvement as well as participating in remyelination in the EAE spinal cord and that it suggests to be effective in therapeutic approaches for MS.

Keywords: Immunologic; Inflammation; Mammalian target of rapamycin; Polyunsaturated fatty acid; Rapamycin

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INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disease associated with neurodegeneration of the brain leading to disability and damage to nerve tissue[1]. Characteristically, Th cell cytokines homeostasis is vital in spleen cell immunity. An imbalance between regulatory and pro-inflammatory cytokines has been involved in the central nervous system (CNS) pathogenesis, mainly MS disease.[2] Mammalian target of rapamycin (mTOR) signaling plays a critical role in neurodevelopmental and neurodegenerative,[3] and the mTOR dysregulation is related to the progression of MS.[4] The mTOR signaling is believed to be a potential therapeutic strategy for EAE.[5] The observations suggest differential regulation of mTOR complexes, mammalian target of rapamy cincomplex 1 (mTORC1) by the regulatory-associated protein of mTOR (RAPTOR), as an essential component and mTORC2 by the regulatory-associated companion of mTOR (RICTOR), as an essential component, is performed on T cell differentiation into T helper (Th) 1, Th2, Th17, and regulatory T (Tregs) cells, and cells function.[6] The pro-inflammatory cytokines of IFN-γ and IL-17 that are released by Th1 and Th17 cells extensively express in the CNS of MS patients and play a critical role in the pathogenesis of EAE, while Treg cells mediate tolerance and reduce inflammation and autoimmune inhibitory status.[7] The signaling of mTORC1 and mTORC2 is susceptible to inhibition by rapamycin (RAPA).[8] Of course, it has been reported that mTORC1 is much more sensitive to RAPA than mTORC2, which is non-sensitive to short time RAPA treatment, however, prolonged treatment to the RAPA can disturb its construction.[9] RAPA ameliorates EAE by preventing Th1 and Th17 cells, as long upregulating forkhead box P3 (FOXP3)-Treg cells[10] that secrete the cytokine inhibitor IL-10.[11] Further, mTORC1 and mTORC2 are involved in the control of polyunsaturated fatty acids (PUFA) and remyelination.[12,13] It has been reported that evening primrose/hemp seed (EP/HS) oil containing ω6/ω3-PUFA as a food/medicine supplement has the potential to reduce the symptoms of MS patients and modulate pro-inflammatory cytokines. EP/HS oil targets the basic mechanism of the disease and works similarly to appropriate treatments in MS.[14] The hemp seed oil (HSO) content of essential fatty acids (EFAs) has a favorable ratio between 2: 1 and 3: 1 of ω6-linoleic acid (LA)/ω3-alpha-linolenic acid (ALA) to repair cell membrane,[15] for example myelin. The HSO exhibits potent anti-oxidative properties that act on specific signaling pathways to modulate inflammatory responses.[16] The gamma-linolenic acid (GLA) content of evening primrose oil (EPO) has inflammatory properties and is recommended for autoimmune conditions, such as MS.[17] The spleen is the major site of T cell activation and immune tolerance induction and plays an essential role in the development of EAE. To explain the immunomodulatory properties of EP/HS oil compared to RAPA, we isolated spleen cells from different groups of mice and evaluated the expression of RAPTOR, RICTOR, IFN-γ, IL-10, STAT3, FOXP3, and IL-17 genes by RT-PCR as well as histological findings.

MATERIALS AND METHODS

Materials

The content of this paper is extracted from the project (NO. 1395-01-00-2835).

Myelin - oligodendrocyte glycoprotein (MOG) $_{35\text{-}55}$ peptide, phosphate buffer saline (PBS), Pertussis toxin and complete freund's adjuvant (CFA) was purchased from Sigma, (3300 S 2nd St #330, St. Louis, MO 63118, USA). RAPA (Sirolimus) was purchased from Santa Cruz Biotechnology (10410 Finnell Street, Dallas, Texas 75220, USA). Ethyl alcohol and formaldehyde were procured from Merck (Mainzer Str. 41, 64579 Gernsheim, Germany). RNeasy and superscript reverse transcriptase kits were purchased from Gene All (303-7, Dangnam-ro, Songpa-gu, Seoul, South Korea). Power SYBR Green real-time polymerase chain reaction (RT-PCR) master mix kit was supplied by Ampliqon (Stenhuggervej 22, 5230 Odense, Denmark).

Mice

Thirty-five young adult C57BL/6 mice (gender: female; age: 6-8 weeks) purchased from Pasteur Institute of Iran, the production and research complex. The animals were kept in an exact pathogen-free situation and handled. Female mice are superior to males for the study of neuropathic studies associated with $MOG_{35\text{-}55}$-induced EAE. $MOG_{35\text{-}55}$ produced behavioral signs of neuropathic hypersensitivity-in females, but not males.[18]

Ethical Statement

All operations were performed according to the Urmia University of Medical Science protocol, Urmia, I.R. Iran (Ethics committee approval No. IR.UMSU.REC.1396.73) and in agreement with animal care.

Experimental Autoimmune Encephalomyelitis Induction

As previously described,[19] MOG_{35-55} peptide solution was prepared by dissolving the peptide inPBS by the concentration of 3 mg/mL. Then, 100 µL of the peptide solution was emulsified with the same volume of CFA (MOG-CFA emulsion). Afterward, 200 µL of MOG-CFA emulsion was injectedinto two flanking sites of each mouse. Besides, pertussis toxin dissolved in PBS and then injected intraperitoneally (i.p) by the concentration of 5 mg/mL immediately and two days later. Mice were monitored for the clinical score of the disease,[20] and daily weights that results were published.[21]

Experimental Animal Groups

After induction of EAE in the C57BL/6 mice, 18 mice were randomly assigned to three groups (EAE/administered) compared to two control groups (EAE and naive) and each group contained 6 mice: Group A. RAPA+EPO/HSO mice subjected to EAE were treated with EPO/HSO (50 µL/mouse)[22] and RAPA (1 mg/kg/50 µL),[23] Group B. RAPA mice subjected to EAE were treated with RAPA (1 mg/kg/50 µL),[23] Group C. EPO/HSO subjected to EAE were treated with EPO/HSO (50 µL/mouse);[20] Group D. EAE control mice subjected to EAE were treated with 1% ethyl alcohol and diluted with distilled water; Group E. Naive group were treated with 1% ethanol-water solution. When the clinical signs of EAE began to appear (the onset of active disease), the mice were treated and continued until sacrifice. Immediately after 14 days post-immunization and onset of disease signs, RAPA was injected daily (i.p) into groups A and B animal, also EPO/HSO was administered orally to groups A and C. Treatments were continued for two weeks.

Treatment of EAE Mice Models with Rapamycin and Hemp Seed/Evening Primrose Oils

Pure hemp seed and evening primrose oils were isolated from the commercial seeds in standard cold-pressed method at Giah Essence Agro-Industry & Phytopharm Company, Gorgan, Golestan Province, 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 ethanol (Merck, Germany) then diluted with purified water and stored at 4°C in the refrigerator following the manufacturer's order. The EAE control solution included 1% ethanol (Merck, Germany) and diluted with distilled water.

Histological Assessment

At the end of the 28th day of study, mice were sacrificed after administration of ketamine/xylazine (i.p. 80/10 mg/kg). Then, the partial vertebral columns were cautiously dissected and incubated overnight at 4°C for post-fixation tissue. The spinal cords were decalcified for two days and washed for 12 hours to remove the decalcification solution. Dehydration of the tissues was done with ethanol solutions then the tissues were fixed in paraffin wax. Then, the spinal cord tissues were sectioned and handled to evaluate the parameters of the disease. The fixed tissues were cut into sections with 6 µm-thickness and prepared for routine staining by hematoxylin and eosin (H&E) for access to infiltration of inflammatory cells and specific staining by luxol fast blue (LFB) for demyelination and acute axonal damage monitoring. Inflammatory lesions and damaged myelin were examined under light microscopy (X400).[24]

Demyelination and inflammation were determined by the presence of spongy tissue and inflammatory cells of the spinal cord in white matter. Sections were observed for inflammatory lesions and demyelination.[24] The resulting in slides in each area of the spinal cord were classified on a 4-point scale. The zone with maximum tissue damage was used to evaluate each spinal cord area.[24]

Preparation of Murine Splenocytes

Spleens isolated from mice. The cells were flushed out of the spleen, gently using a syringe plunger with sterile normal saline and transferred to the falcon tubes and then centrifuged for 5 minutes (2000 rpm, 4⁰C) and aspirated the supernatant. The pellets were suspended in ammonium chloride solutions (0.9%) to eliminate erythrocytes. After further centrifugation, the supernatants were discarded and resuspended in normal saline at "2×10^6" cells per mL. One million cells in a

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volume of 0.5 mL have been used to perform the following steps.

Quantitative Real-time Polymerase Chain Reaction

The transcript level of inflammatory cytokines and immunological factors was quantified by quantitative real-time polymerase chain reaction (qRT-PCR). Murine splenocytes were harvested as described in Methods. Briefly, total RNA was prepared separately from splenocytes samples using random hexamer primers and reverse transcriptase kit (Gene All, South Korea) according to the company's protocol. The extracted RNA purity was evaluated by estimating the optical density ratio at 260 to 280 nm. RNA integrity was also assessed by agarose gel electrophoresis.

Two µg of total RNA was subjected to reverse transcription then converted into cDNA using the first-strand cDNA synthesis kit (Gene All, South Korea). The PCR mixture was prepared using the Power SYBR Green qPCR Master Mix kit (Ampliqon, Denmark). Real-time PCR with gene-specific primers set RAPTOR, RICTOR, IFN-γ, IL-10, FOXP3, STAT3, IL-17, and β-actin2 was performed based on the manufacturer's protocols (Bio-Rad iQ5 cycler sequence detection system). A negative control (without cDNA) was used to investigate unwanted contamination and primer dimer formation. Reaction mixtures were performed in the following condition: initial denaturation; 95⁰C for 51s, annealing; 60.5⁰C for the 60s and β-actin2 was used as an internal control. RNA integrity used for real-time PCR was confirmed by β-actin2 synthesis as a positive control reaction. Sequences of primers are shown in Table 2.

To confirm the specificity of amplification reactions, melt curve analysis was performed. For relative quantification calculation, the $2^{-\Delta\Delta CT}$ formula was used.[25] The amplified qRT-PCR products presented in Figure 1.

Table 1. Fatty acid profiles (%) of hemp seed and evening primrose oils by gas chromatography. ALA: alpha-Linolenic acid; PUFA: Polyunsaturated fatty acids (ω6/ω3-PUFAs); SDA: Stearidonic acid; GLA: gamma-linolenic acid

Seed Oil	Palmitic acid	Stearic acid	Oleic acid	Linoleic	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**

Table 2. Sequences of primers to evaluate the expression of mTORC1 (RAPTOR), mTORC2 (RICTOR), IFN-γ, IL-10, FOXP3, STAT3, IL-17 and β-actin genes in spleen cells

Target gene		Primer sequence	Product Size (bp)
β-actin	Forward	5´- CGTTGACATCCGTAAAGACC -3´	285
	Reverse	5´- CAGTAACAGTCCGCCTAGAA -3´	
mTORC1 (†RAPTOR)	Forward	5´- ACAGCCATCTACAACGGAAA -3´	179
	Reverse	5´- TATCTGACACGCTTCTCCAC -3´	
IFN-γ	Forward	5'- AACTGGCAAAAGGATGGTGA -3´	153
	Reverse	5'- GCTGTTGCTGAAGAAGGTAG -3'	
mTORC2 (‡RICTOR)	Forward	5´- GGAGCACACGGATGACAAT -3´	153
	Reverse	5´- TCTAAGGGGTTGTGGATCGT -3´	
IL-10	Forward	5'- CCCTTTGCTATGGTGTCCTT -3´	185
	Reverse	5'- GCCACAGTTTTCAGGGATGA-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'	

† RAPTOR, an essential component of mTORC1, ‡RICTOR, an essential component of mTORC2

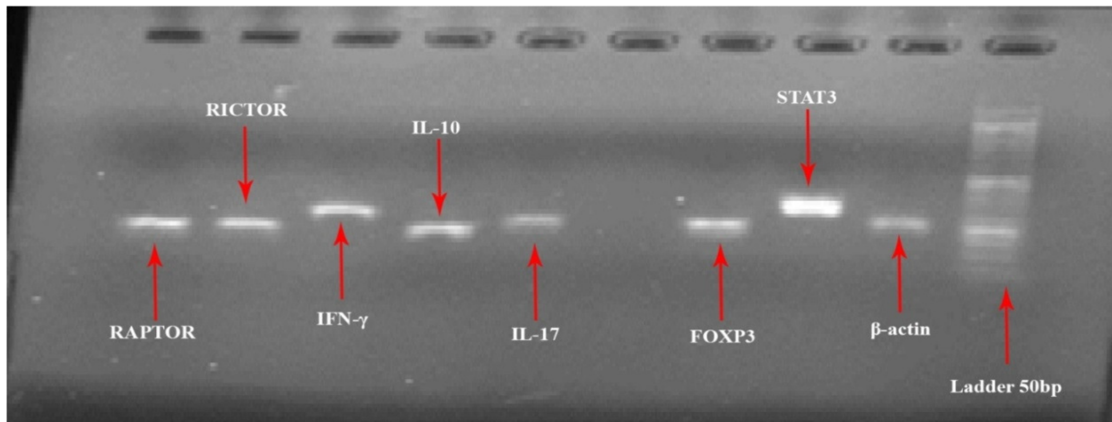

Figure 1. The amplified quantitative real-time polymerase chain reaction products of RAPTOR-mTORC1, RICTOR-mTORC2, IFN-γ, IL-10, IL-17, FOXP3, STAT3, and β-actin2 that were evaluated by agarose gel electrophoresis with ladder 50bp (left to right).RAPTOR-mTORC1, Regulatory-associated protein of mammalian target of rapamycin, RICTOR-mTORC2, regulatory-associated companion of mammalian target of rapamycin, IFN-γ, interferon-gamma, IL-10, interleukin-10, IL-17, interleukin-17, FOXP3, forkhead box P3, STAT3, signal transducer and activator of transcription factors.

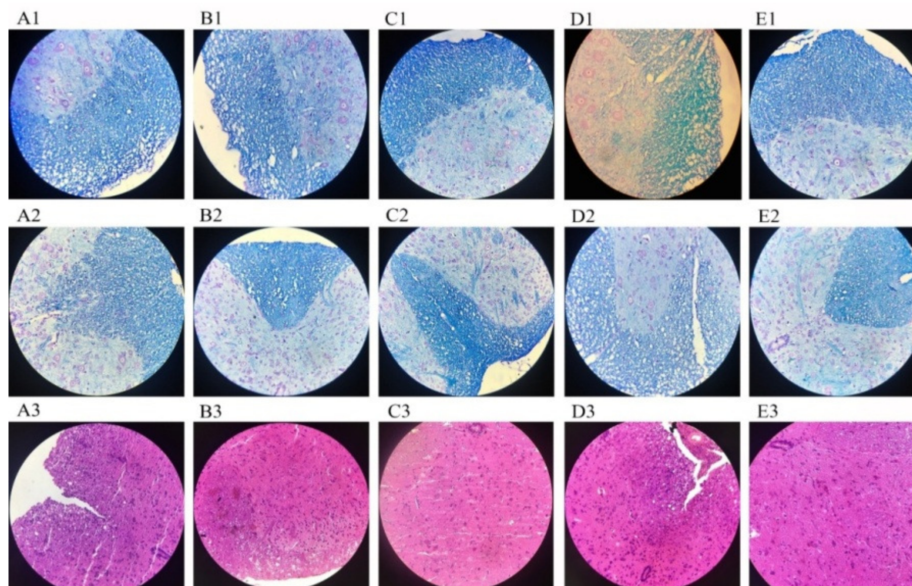

Figure 2. The effects of HSO/EPO and/or RAPA treatments on cellular infiltration and demyelination in the spinal cord of EAE mice. Spinal cords sections were stained by H&E (for cellular infiltration) and LFB (for demyelination). Each column represents different sections of the spinal cord of one mouse. The results showed that EPO/HSO treated mice (C1-C3) show a few focal inflammatory cells, but not degenerative neurons and spongy lesions in white matter. ANOVA followed by Tukey's post hoc test performed for statistical analysis (N=5 mice in each group, *p*<0.05). (A1-A3, RAPA+EPO/HSO; B1-B3, RAPA; D1-D3, EAE; E1-E3, Navie). LFB, luxol fast blue; H & E, hematoxylin and eosin; EPO/HSO, evening primrose oil/hemp seed oil; RAPA, rapamycin, ANOVA, One-way analysis of variance, EAE, experimental autoimmune encephalomyelitis, Magnification=400.

Statistical Analyses

One-way analysis of variance and Tukey post hoc test was used for statistical analysis using SPSS software (Version 20). qRT-PCR results of all experiments were repeated in duplicate. The mean±standard error of the mean (SEM) was used for data. A $p \leq 0.05$ was measured as statistically significant.

RESULTS

The EPO/HSO, RAPA, and RAPA+EPO/HSO groups were compared to the two control groups (EAE and naïve mice) as described in Materials and Methods. All EAE mice exhibited chronic disease. The EPO/HSO treatment of mice significantly reduced EAE severity concerning various statistical parameters of EAE. Thus, these results suggest that the EPO/HSO applied by oral route ameliorated EAE in mice.[21]

Histological Assessment of the Spinal Cord

The histological evaluations of the spinal cord are shown in Figure 2. On the 28th day after immunization, severe infiltration of numerous inflammatory cells was identified in the spinal cord in RAPA+EPO/HSO and EAE mice groups, which showed an incidence of immunological function. In the spinal cord of mice treated with EPO/HSO infiltrated only a few inflammatory cells that showed inhibition of inflammation. As for the EPO/HSO group, no significant abnormalities were identified in the spinal cord. In the RAPA group, the anti-inflammatory effects were significantly reduced by the following treatment, whereas the RAPA+EPO/HSO was least affected.

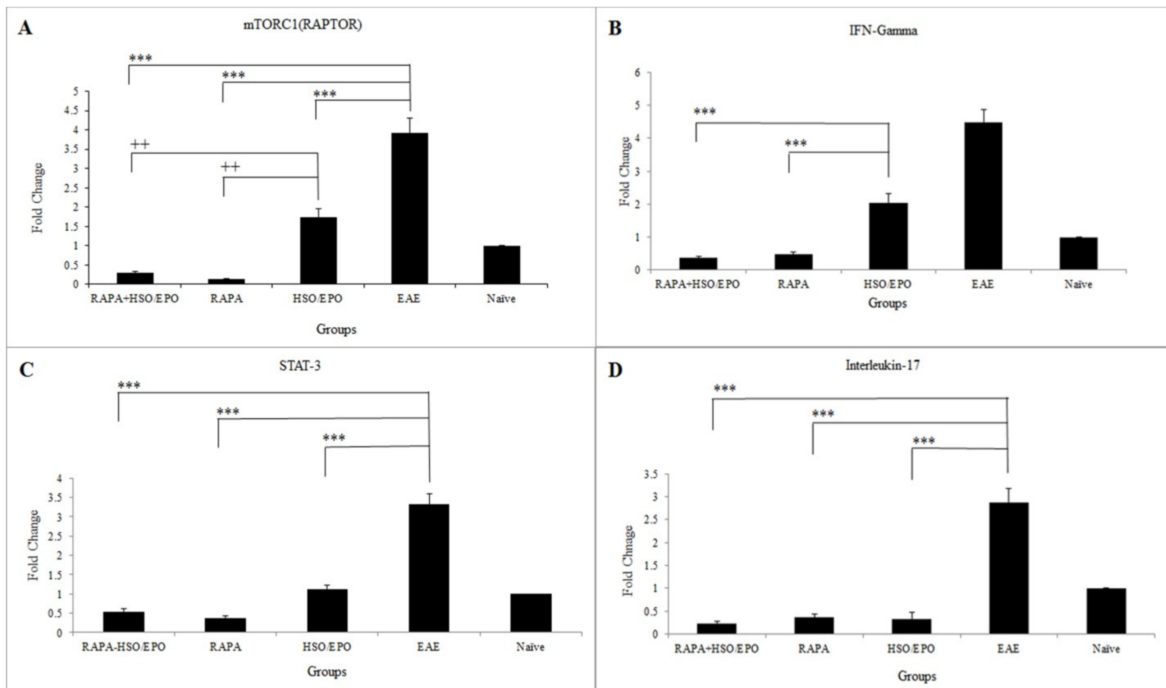

Figure 3. The effects of HSO/EPO and/or RAPA treatment on RAPTOR-mTORC1 (A), IFN-γ(B), STAT3(C), and IL-17(D) gene expression in EAE mice. RNA extraction from splenocytes and qRT-PCR was performed to evaluate the expression level of the mentioned genes in EAE suffering mice. The results showed that treatment with HSO/EPO resulted in a significant reduction in mTORC1, IFN-γ, STAT3, and IL-17 gene expression compared to the EAE group. ANOVA followed by the Tukey post hoc test was used to perform statistical analysis (N=5 mice in each group). Data are shown as mean±SEM. *** $p<0.001$ compared to the EAE group. ++ $p<0.05$ compared to HSO/EPO group.RAPTOR-mTORC1, Regulatory-associated protein of mammalian target of rapamycin, IFN-γ, interferon–gamma, IL-17, interleukin-17, STAT3, signal transducer and activator of transcription factors. HSO/EPO, evening primrose/hemp seed oil; RAPA, Rapamycin; EAE, experimental autoimmune encephalomyelitis; ANOVA, One-way analysis of variance.

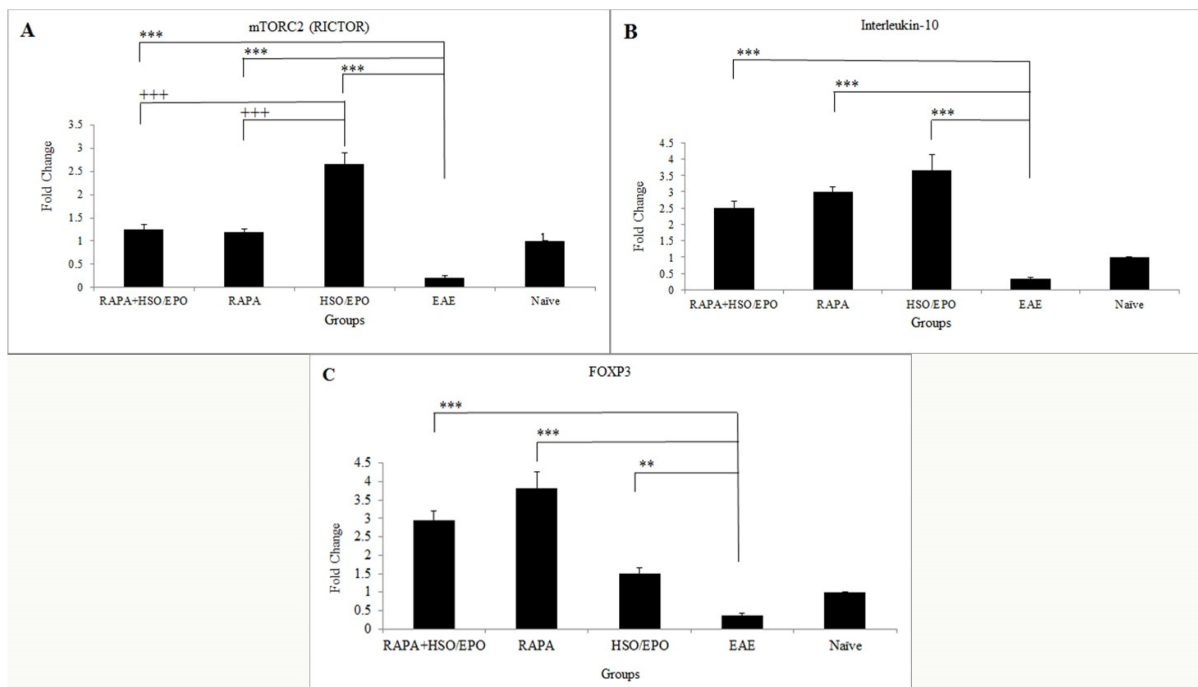

Figure 4. The effects of HSO/EPO and/or RAPA treatment on RICTOR-mTORC2(A), IL-10(B), and FOXP3(C) gene expression in EAE mice. RNA extraction from splenocytes and RT-PCR was performed to evaluate the expression levels of the mentioned genes in EAE suffering mice. The results showed that treatment with HSO/EPO increased mTORC1, IFN-γ, FOXP3, and IL-17 gene expression compared to the EAE group. ANOVA followed by Tukey's post hoc test wasused to perform statistical analysis (N=5 mice in each group). Data are shown as mean±SEM. *** $p<0.001$, ** $p<0.05$ compared to EAE group, +++ $p<0.001$ compared to HSO/EPO group.RICTOR-mTORC2, regulatory-associated companion of mammalian target of rapamycin, IL-10, interleukin-10, FOXP3, forkhead box P3, HSO/EPO, evening primrose/hemp seed oil; RAPA, Rapamycin; EAE, experimental autoimmune encephalomyelitis, RT-PCR, real time-polymerase chain reaction, ANOVA, One-way analysis of variance.

Expression of RAPTOR, RICTOR, IFN-γ, IL-10, IL-17, FOXP3, and STAT3 Genes

To investigate the transcriptional effects of inflammation mediators in the EPO/HSO group compared with other groups, expression levels of RAPTOR-mTORC1, RICTOR-mTORC2, IFN-γ, IL-10, IL-17, FOXP3, and STAT3 genes in spleen cells of all groups were analyzed. In the HSO / EPO group, a statistically significant reduction in the expression of RAPTOR-mTORC1, IFN-γ, STAT3, and IL-17 genes, as well as upregulation of RICTOR-mTORC2, IL-10, and FOXP3 was found in comparison with the EAE group. Also, the results showed complete suppression of RAPTOR-mTORC1, IFN-γ, IL-17, and STAT3 genes in RAPA groups (Figure 4), whereas there was a significant increase in IL-10 and FOXP3, although no difference in RICTOR- mTORC2 compared to the naive group. In contrast to RAPA, results indicated that HSO/EPO controls and modulates inflammation without damaging mTORC1 coordinating cell activities. Besides, similar to other studies, RAPA severely suppressed the expression of RAPTOR-mTORC1 and STAT3 as well as pro-inflammatory cytokines of IL-17 and IFN-γ. Unexpectedly, the increase in the expression of IL-10 and FOXP3 without a significant increase in mTORC2[9]in RAPA groups is due to the proliferation of Treg cells. Thus, the induction of IL-10 did not result from the expansion of Th2 cells.[26]

DISCUSSION

In the present study, we found that in RAPA groups (Figure 3, 4), suppression of RAPTOR, the increasing

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of FOXP3 and anti-inflammatory cytokine IL-10, indicate that RAPA plays an important anti-inflammatory role by induction of tolerance. Tolerance is reduced by deletion and induction of anergy of activated T cells, or by induction of suppressive FOXP3 (a specific marker and major transcription factor in Treg cells) in diseases such as MS/EAE.[27] This change shows IL-10 can be tolerogenic and is required to maintain FOXP3 expression,[33] and is accompanied by a decrease in STAT3 that reduces Th17 cell formation and IL-17 production[34] in RAPA groups.This implies that the inhibitory effect of RAPA on mTOR activation in spleen cells works by restoring the differential balance between Th17 and Treg cells. These results (Figure 3, 4) indicate that the inhibitory effect of RAPA on STAT3-mTOR directs FOXP3 regulatory factor production and prevents IL-17 production in splenocytes, resulting in delayed EAE in RAPA groups. STAT3 is the most important regulator for the pathogenicity of T cells. It is activated at various levels, including the differentiation of Th17 and the production of cytokines, such as IL-17. Analysis of gene expression results (Figure 3, 4) showed that STAT3 and mTORC1 pathways activities were enhanced in EAE control mice due to tissue inflammation, which showed cross-over between mTORC1 and STAT3 pathways in splenocytes.[35,36] Polyunsaturated fatty acids (PUFAs) with their balanced composition of omega 6/omega 3 in a ratio of less than 4 to 1 can modulate immune responses, affecting the function of the CNS cells. Dietary antioxidants can also support the cellular defense system in a variety of ways, including free radical scavenging (ROS), interference with gene transcription, mRNA expression, enzyme activity, and chelating oxidative factors and metals involved in the oxidation process. Thus, both dietary antioxidants and PUFAs can reduce disease symptoms by targeting specific mechanisms and supporting the recovery process in MS/EAE diseases.[15] Also, results in (Figure 3, 4) show that in the EPO/HSO group enhancement of IL-10 and FOXP3 may be due to differential downregulation of pro-inflammatory markers, which would subsequently favor Th2 induction and Treg cells. It means that EPO/HSO alter spleen immune cells and significantly improve the clinical score[21] compared to RAPA groups. With complementary roles in the response to metabolic signals, two mTOR complexes enable the management of triglycerides by stimulating adipogenesis and lipogenesis.[27] Due to the structurally and fundamentally roles of mTORC1 in metabolic and physiological processes and the role of RAPTOR in Th2 cell differentiation, the complete suppression of the mTORC1 is not desirable, as occurred in the RAPA groups.[28] Early studies have shown that mTORC2 signaling plays a key role in CNS myelination. Our data showed that treatment with EPO/HSO increased expression of RAPTOR-mTORC2 and myelination (Figure 2) on based histological observations in the spinal cord sections and confirmed the EPO/HSO efficacy in protecting mice against MOG-induced EAE. Additionally, limited histologic modifications were seen in the tissues of sections taken from the EPO/HSO group, whereas the white matter RAPA group had a wide area of infiltration of the inflammatory cells (Figure 2). The EPO/HSO treatment recovered EAE development by decreasing inflammatory cell infiltration, providing a protective effect on the CNS nerves. The mechanism by EPO/HSO exerts its beneficial effects is not limited to attenuating inflammation by a variety of tocopherols, terpenes and phenolic compounds including phytosterols with potent antioxidant properties, but is also related to its effects on phospholipid synthesis in cell membranes.[14] Consequently, improved expression of the RICTOR-mTORC2 gene after treatment with EPO/HSO may be a cause of improved disease, possibly resulting in stopping the loss and repair of myelin. We observed enhanced expression of mTORC2 in the EPO/HSO group compared to the RAPA groups, whereas co-administration of RAPA and EPO/HSO was not effective as EPO/HSO, mainly due to RAPA's deleterious effects on mTORC1 natural functions. The mTORC1 signaling plays an essential role in the long-standing regulation of lipid homeostasis. Reduced mTORC1 activity increases lipolysis and decreases mitochondrial combustion of free fatty acids.[29] Evidence suggests that chronic inhibition of mTORC1 by RAPA leads to exacerbation of hyperglycemia and insulin resistance that results in an increase in fatty acids in the bloodstream and causes a condition similar to metabolic syndrome.[30] The results showed that EPO/HSO activates the mTORC2 pathway and elevates IL-10 expression that can ameliorate the expression of pro-inflammatory cytokines such as IFN-γ. The mTORC2 pathway is involved in suppressing autoimmune disease progression,[31] as a result, EPO/HSO could induce immunosuppression through

mTORC2 pathway modulation by increased expression of RICTOR-mTORC2, as well as IL-10, and FOXP3 genes compared to RAPA in the EAE model. Also, the reduction of pro-inflammatory factors by EPO/HSO so that expression of Th1 cytokines attenuated. These results are consistent with the report that phosphorylation of mTORC2 leads to inhibition of demyelination.[32] Therefore, the data suggest that the significant potential immunomodulatory effects of EPO/HSO are greater than the immunosuppressive capacity of RAPA, and indicate that EPO/HSO-based strategies may be an alternative promising to MS management.The limitations of the study are small sample size, lack of prior research studies on the topic and funding of research.

In conclusion, it could indicate that since mTOR pathway is a major regulator of nutrient-sensing signals in all mammalian cells 33and the mTOR dysregulation is related to the progression of MS,[4] these results demonstrate the immunomodulatory effects of HSO/EPO on EAE, as well as improved remyelination, which results in the elimination of the immune response. In contrast, treatment with RAPA alone suppressed EAE promotion with low amelioration that was achieved by promoting the proliferation of Treg cells and stopping the generation of Th1 and Th17 cells without significant histological improvement due to mTOR impairment.

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