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The effect of 1, 25(OH)2 D3 (calcitriol) alone and in combination with all-trans retinoic acid on ROR-γt, IL-17, TGF-β, and FOXP3 gene expression in experimental autoimmune encephalomyelitis

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Objectives: It has been shown that calcitriol and all-trans retinoic acid (ATRA) have modulatory effects on the immune system. The present study investigates the synergistic effects of combination treatment of calcitriol and ATRA in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS).

Methods: The mice were allocated to four preventive groups, each consisting of eight animals, ATRA (250 μg/mouse), calcitriol (100 ng/mouse), combination of ATRA and calcitriol (125 μg/mouse and 50 ng/mouse) and vehicle groups. EAE was induced by MOG35-55 peptide in female C57BL/6 mice. Treatments were initiated at day 1 before immunization and continued every other day throughout the study until the day 21 post-immunization. Splenocytes were isolated from EAE-induced mice and the expression of retinoic acid receptor-related orphan receptor gamma t (ROR-γt), Interleukin-17 (IL-17), transforming growth factor beta (TGF-β), and forkhead box P3 (FOXP3) genes was measured using real-time polymerase chain reaction.

Results: The expression of FOXP3 and TGF-β genes in the splenocytes of combination-treated and calcitriol alone-treated mice was significantly increased compared to vehicle group (P < 0.05). The expression of ROR-γt and IL-17 genes in the splenocytes of ATRA, calcitriol and combination-treated mice was significantly reduced compared to those of vehicle-treated mice (P < 0.05). The relative expression level of ROR-γt was significantly (P < 0.05) lower in the combination group than in the mice treated by ATRA or calcitriol alone.

Discussion: This study demonstrated that treatment with combination of calcitriol and ATRA can be considered as a new strategy for MS prevention and treatment.

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Introduction

Multiple sclerosis (MS) is a chronic inflammatory and demyelinating autoimmune disease of the central nervous system (CNS). The etiology of MS is still unclear; however, it is supposed to result from an aberrant response of the immune system to one or more myelin antigens. MS is a CD4+ T cell-mediated autoimmune disease in which naive CD4+ T cells differentiate into proinflammatory helper T cells; therefore, in these patients the frequency of Th1 and Th17 cells is increased, while the population of immune-protective regulatory T (Treg) cells is decreased. This imbalance is thought to play a crucial role in nerve damage in MS. Studies have shown that ROR-γt is the key transcription factor essential for Th17 cell differentiation. Th17 cells have been illustrated as a novel subset of the specialized lineage of Th cells that produces IL-17. Th17 cells are potent inducers of tissue inflammation and have been associated with the pathogenesis of many experimental autoimmune diseases and human inflammatory conditions. The role of Th17 cells in autoimmunity was demonstrated first in mice that were deficient for the p19 chain of the IL-23, in which the IL-17-producing T cells were significantly lower than in wild-type mice, highlighting the importance of the IL-23/Th17 axis in the pathogenicity of these autoimmune diseases. Since then, the study of the pathogenic role of Th17 subset cells has focused on autoimmune inflammatory diseases, such as multiple sclerosis, rheumatoid arthritis, and psoriasis. Effector cytokines of Th17 cells, including IL-17, IL-6, IL-21, IL-22, and IL-23 have proinflammatory properties that are involved in the immunopathogenesis of MS. In a variety of human studies, Th17 cells have been detected in tissues of MS patients. The adverse effect of IL-17 in MS has been mentioned in several studies. Human blood–brain barrier (BBB) endothelial cells from MS patients express the receptors for IL-17, and exposure of these cells to IL-17 results in low expression of tight junction proteins and increased transmigration of CD4+ T cells into the brain. Moreover, IL-17 elevates expression of matrix metalloproteinase expression, leading to BBB dysfunction and neuronal apoptosis. Thus, the repression of development or proliferation of Th17 cells might designate a promising therapy for MS. A number of nutritional factors such as retinoids can modulate the maturation process of these cells. Moreover, FOXP3 + Treg cells play an important role in the maintenance of immune system homeostasis and immunological self-tolerance. As studies have shown in the individuals with MS, the proliferation and cytokine secretion of Treg cells are impaired and the level of FOXP3 expression was decreased. Cytokines produced by Treg cells, including IL-10, IL-35, and TGF-β, have immunosuppressive features and can suppress the differentiation of naive CD4+ T cells into pathogenic Th17 cells. Treg cells produce high amount of TGF-β that suppresses proinflammatory cytokines secreted by Th17 cells. TGF-β and retinoic acid (RA) can induce Treg cell development. In MS patients, retinoic acid (the active metabolite of vitamin A) suppresses immunopathogenic pathways through the upregulation of FOXP3 + Treg cells and their differentiation. Vitamin A also down regulates ROR-γt and IL-17 gene expression levels in MS patients as we demonstrated previously. Vitamin D is a known nutrient that plays an essential role in calcium homeostasis and acts as an immune system modulator. Extensive evidence suggests that insufficient vitamin D supply and diminished synthesis of calcitriol contributes causally to frequent MS relapses, and rapid MS disease progression. Furthermore, accumulating evidence shows that the vitamin D endocrine system promotes vascular health and neuroprotective functions, including keeping of memory and cognition, neurotransmission, and neuroplasticity. Vitamin D receptor is expressed on a number of immune cell types, including monocytes, macrophages, dendritic cells (DCs), and effector/memory T cells. T cell proliferation, generation of IL-2 and IFN-γ and its
cytotoxicity effect, is suppressed by 1, 25(OH)2 D3 in *in vitro* studies.32 1, 25(OH)2 D3 through reducing expression level of the MHC class II and CD40 negatively regulates the differentiation, maturation, and immunostimulatory capacity of DCs.33 It also differentially regulates the differentiation, maturation, and expression level of the MHC class II and CD40 negatively regulates the differentiation, maturation, and immunostimulatory capacity of DCs.33 It also probably inhibits the generation of Th1 and Th17 cells and stimulates the development of FOXP3+ Treg cells.26 A study of select representatives of the Th17 transcriptome showed that the levels of mRNAs that encode ROR-γt, IL-17A, IL-17F, IL-23R, and IL-22, were reduced by 1, 25(OH)2 D3.35 Beneficial effects of calcitriol treatment were observed in a small pilot study in relapsing remitting MS patients.36 Experimental autoimmune encephalomyelitis (EAE) is the most commonly used animal model for MS. Several currently approved MS therapeutics, natalizumab, mitoxantrone, and copaxone, were developed based on EAE studies.37 However, there is limited information about the role of combined treatment of 1, 25(OH)2 D3 and ATRA on MS patients and EAE-induced mice. In order to investigate new potential therapeutic targets in MS and other inflammatory demyelination diseases, such as Devic’s disease, chronic relapsing inflammatory optic neuritis, acute disseminated encephalomyelitis (ADEM), and Balo concentric sclerosis, the present study investigated the effect of ATRA, 1, 25(OH)2 D3 and their combined treatment on the expression of IL-17, ROR-γt, TGF-β, and FOXP3 genes in the EAE-induced mice.

**Materials and methods**

**Animals**

Inbred female C57Bl/6 mice, 9–10 weeks old, were purchased from Iran Pasteur Institute (Pasteur’s Institute, Tehran, Iran). The mice were maintained at 23 ± 1°C and 12:12 hours light: dark cycles. Water and food was provided ad libitum. Experiments were performed in compliance with the Policies of animals research in neuroscience.38 The study protocol has been approved by the ethics committee of Tehran University of Medical Sciences.

**EAE induction and clinical evaluation**

After a week acclimation, 32 animals were immunized with Hooke kits (Hooke labs, EK-2110, Lawrence, MA, USA) in accordance with manufacturer’s instructions. In brief, after deep anesthesia with ketamine/ xylazine, two 100 µl MOG/CFA emulsion was injected subcutaneously into two sites of the flanks of each mouse. Two and 24 hours after immunization, the mice were injected intraperitoneally with 200 ng of pertussis toxin diluted in 100 µl PBS. Clinical scores were recorded daily by two researchers who were blinded to the treatment groups as follows: 0, no clinical disease; 0.5, partial tail paralysis; 1.0, complete tail paralysis or limp tail; 1.5, complete tail paralysis and partial paralysis one hind limb; 2.0, complete tail paralysis and partial paralysis of both hind limbs; 2.5, partial paralysis of one hind limb and complete paralysis of one hind limb; 3.0, paralysis of both hind limbs without forelimb weakness; 4.0, hind and one forelimb paralysis; 5.0, moribund/dead.39 Before scoring, the mice weights were recorded every day. Mean clinical score was computed by summing the daily clinical scores for all mice in a group then divided by the total number of mice. Maximum mean clinical score (MMCS) was the mean clinical score at the peak of disease. Average mean clinical score (AMCS) was calculated by adding the mean clinical score and then divided by total days. The cumulative disease index (CDI) was calculated by summing each animal’s daily EAE clinical score during 21 days.

**Treatment of mice**

EAE-induced mice were randomly assigned to four experimental groups consisting eight mice in each: ATRA, calcitriol, combination of ATRA and calcitriol and vehicle-treated groups. All treatments were started at 1 day before the immunization and were administered via intraperitoneal (i.p.) injection every other day. Calcitriol (Kern Pharma, Spain) was administrated to the calcitriol-treated group at a dose of 100 ng per mouse. ATRA (Sigma-Aldrich, St. Louis, MO, USA) was injected to ATRA-treated group at a dose of 250 µg per mouse. Each mouse of combination-treated group received 50 ng of calcitriol and 125 µg of ATRA (i.e. half dose of calcitriol and ATRA in single treatments). Vehicle-treated mice received an equal volume of excipient. The doses in this study were selected based on previous studies.37,40–44 Furthermore, the high dose of these nutrients might have potential toxicological effects39,45 so using half dose of each nutrient seems appropriate.

**RNA extraction and real-time polymerase chain reaction**

Twenty-one days after the induction of EAE, spleens were aseptically removed from mice and single-cell suspensions of splenocytes were obtained as previously described.46 Briefly, the cells were centrifuged at 1500 rev min−1 for 5 minutes and after removing the supernatant, the cells were incubated with 3 ml ACK (ammonium–chloride–potassium) lysing buffer for 5 minutes at room temperature for lysis of red blood cell. The resulting mixture was centrifuged at 1500 rev min−1 for 5 minutes and the supernatant was removed. The remaining cell suspensions were the spleen mononuclear cells (SMCs). Total RNA
was extracted and purified from SMC pellets by RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. Quality and purity of the extracted RNA was assessed by a spectrophotometer (NanoDrop, Thermo Scientific). The prepared RNAs from all samples were reverse-transcribed using a cDNA synthesis kit (Takara Bio, Inc., Japan). Standard quantitative real-time polymerase chain reaction (PCR) was carried out in Step One System (Applied Biosystems, Foster City) using the SYBR Green method (Takara Bio, Inc., Japan). The NCBI tool Primer BLAST was used to design primers. The sequences of primers are shown in Table 1. The GAPDH was used as housekeeping gene.

Statistical analysis
Data are presented as mean ± S.E.M and analyzed by the SPSS 22.0 software. The Kolmogorov–Smirnov test was used for evaluating normal distribution. For normal distributed data, one-way ANOVA and for non-normal distributed data, Kruskal–Wallis, and Mann–Whitney U test were used. Differences between groups were considered statistically significant when P values were less than 0.05.

Results
Clinical follow-up
In order to evaluate the prophylactic effect of ATRA and calcitriol, the EAE- induced mice were treated by these vitamins 1 day before the immunization. As shown in Table 2, MMCS and AMCS were significantly reduced in combined and calcitriol- treated mice compared to vehicle-treated group. In addition, AMCS in mice received half doses of ATRA and calcitriol in combination was obtained significantly less than ATRA group (Table 2). The results showed no statistically significant difference between the combination treatment with calcitriol plus ATRA and calcitriol alone. Statistical analysis of clinical scores showed that treatment with combination of calcitriol and ATRA led to significant reduction of disease disability from day 13, compared to vehicle-treated group. This period was started from day 16 in calcitriol-treated animals but was not found in ATRA- treated group (Fig. 1).

FOXP3 and TGF-β gene expression in the freshly isolated splenocytes
We examined the levels of FOXP3 and TGF-β mRNA expression in the splenocytes obtained from the EAE-induced mice treated with ATRA, calcitriol, combination of ATRA plus calcitriol, and vehicle. The expression levels of FOXP3 and TGF-β in the splenocytes of combined and calcitriol- treated mice were significantly increased as compared with that of vehicle group (Figs. 2 and 3). In ATRA group, the expression levels of FOXP3 and TGF-β genes showed no significant differences compared with that in vehicle group. In addition, the relative expression levels of FOXP3 and TGF-β were higher in combination and calcitriol group than those in the ATRA group (Figs. 2 and 3).

ROR-γt and IL-17 gene expression in the freshly isolated splenocytes
The results of ROR-γt and IL-17 gene expression in the splenocytes of EAE-induced mice are shown in Figs. 4 and 5, respectively. The expression levels of ROR-γt and IL-17 genes in ATRA-treated, calcitriol-treated, and combined-treated mice were significantly down regulated compared to those of vehicle groups. In addition, the relative expression levels of ROR-γt were significantly lower in the combined-treated group than the ATRA and the calcitriol groups (Fig. 4). IL-17 relative expression was also significantly decreased in calcitriol compared to ATRA group (Fig. 5).

Discussion
In the present study, the impacts of ATRA alone, calcitriol alone, and their combination on the mRNA expression of IL-17, ROR-γt, TGF-β, and FOXP3 in splenocytes of EAE- induced mice, were explored. Since the clinical signs of EAE usually begin between day 9 and 14 post-immunization, vitamin administration at the same time with immunization or a few days before immunization can be considered preventive. According to previous studies, all treatments in the present study were started at 1 day before the immunization.26,48,49 Recent studies have shown that nutrients, especially vitamins, have important roles in immune system function.11,24,25,50 The main nutritional disadvantage of fat- soluble vitamin therapy may be the fact that these vitamins have shown their effects at high pharmacological doses which can be toxic and may lead to adverse effects. For example, hypercalcemia is a dose-limiting effect that prevents sustained systemic administration of 1, 25(OH)2 D3 and leukocytosis is an adverse effect in APL treatment by ATRA.39,45 One solution to overcome this problem would be to use the combination of these vitamins in lower doses (e.g. half dose or less). Discovery of a synergistic effect at lower doses would be very useful to reduce side effects and increase the efficacy of treatment. To our knowledge, it is the first study to examine the effects of combination treatment of ATRA and calcitriol on the expression of the above mentioned genes in EAE-induced mice. Our study showed that the gene expression of these cytokines and transcription factors was changed in treatment groups compared to vehicle groups. The results of our study demonstrated that gene expression of ROR-γt was significantly decreased in ATRA,
calcitriol, and combination groups in comparison with control group (P < 0.05, Fig. 4). Moreover gene expression of ROR-γt was significantly down regulated in combination group compared to ATRA and calcitriol alone groups. Considering that concentration of ATRA and calcitriol in combination group was half of the concentration treated in individual groups, these two vitamin derivatives could synergistically decrease ROR-γt gene expression in combination compared with individual treatments (Fig. 4). ROR-γt is Th17 lineage- specific transcription factor and plays a critical role on Th17 differentiation and IL-17 production.51 Mice with defect in ROR-γt are resistant to EAE induction.51 The results of this study showed that interleukin-17 gene expression in all intervention groups significantly down regulated compared to control group. There were no significant differences between combination and alone groups. Therefore, we were able to achieve the same results in combination treatment with half dose compared to full dose in single treatment. In line with our findings, RA has been shown to ameliorate EAE.52 ATRA has been reported to be effective in the downregulation of ROR-γt and reduction of IL-17 production.53 Possible mechanisms of action of ATRA in suppression of Th17 cell function have been reported to be a result of reduced expression of IL-6 receptor and IL-23 receptor as well as enhanced TGF-β signaling in a Smad3-dependent manner followed by the downregulation of ROR-γt and the decreased production and differentiation of Th17 cells.54 Disease prevention studies in animal models have also suggested that calcitriol directly inhibits encephalitogenic Th17 cells. In experimental autoimmune uveitis, in vivo calcitriol treatment of mice impaired T-cell commitment to the Th17 lineage as well as Th17 production of IL-17.55 Similar results were reported in the EAE model. The spleens of calcitriol-treated mice had fewer splenic Th17 cells and lower IL-17 production than the placebo controls.26,50 Calcitriol treatment reduced Th17 cells in the CNS in an EAE prevention study56 and in two EAE treatment studies.39,41 Different mechanisms were suggested to explain these findings. In a study by Chang et al.50, calcitriol did not suppress IL-17 gene transcription, but inhibited IL-17 production in a VDR-dependent manner by a post-transcriptional mechanism. Joshi et al.41 showed that calcitriol suppressed IL-17 gene transcription by blocking nuclear factor for activated T cells, recruiting histone deacetylase, and sequestering Runt-related transcription factor1 (Runx1). In another study, Nashold et al. (2013) suggested that Th17 cells were eliminated by a programmed cell death mechanism.39 According to our findings, it has been shown that calcitriol blocks IL-17A expression by reducing the expression of ROR-γt mRNA.35 Our data showed that gene expression of FOXP3 and TGF-β was significantly higher in calcitriol group and in combination group than in control group. However, ATRA and vehicle groups showed no significant differences. In addition, gene expression levels of FOXP3 and TGF-β were significantly higher in calcitriol and combination groups than in ATRA group. There were no significant differences in the expression level of FOXP3 and TGF-β between calcitriol and combination group. Regulatory T cells are essential for maintaining immune system homeostasis by promoting self-tolerance and restraining excessive

### Table 1 Characteristics of quantitative real-time PCR primers

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Sequence</th>
<th>Length</th>
<th>Tm</th>
<th>CG%</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOXP3-F</td>
<td>5′-CCTGCTTGTGATCCATGTC-3′</td>
<td>20</td>
<td>60.5</td>
<td>55</td>
</tr>
<tr>
<td>FOXP3-R</td>
<td>5′-TGTGTTGGTGGAGCTGTTG-3′</td>
<td>20</td>
<td>58.4</td>
<td>50</td>
</tr>
<tr>
<td>TGF-β-F</td>
<td>5′-ATTGCTTCACGTCCACAGAG-3′</td>
<td>20</td>
<td>58.4</td>
<td>50</td>
</tr>
<tr>
<td>TGF-β-R</td>
<td>5′-TGACTTGTGTTGGAGCTGCC-3′</td>
<td>20</td>
<td>60.5</td>
<td>55</td>
</tr>
<tr>
<td>IL-17-F</td>
<td>5′-GCTCCAGAAGGGGCCTCAGA-3′</td>
<td>19</td>
<td>61.7</td>
<td>63.16</td>
</tr>
<tr>
<td>IL-17-R</td>
<td>5′-AGCTTCCCTCAGGATTGA-3′</td>
<td>19</td>
<td>57.3</td>
<td>52.63</td>
</tr>
<tr>
<td>ROR-γt-F</td>
<td>5′-CAAGTCACTGGGATCCACTAC-3′</td>
<td>23</td>
<td>62.9</td>
<td>47.83</td>
</tr>
<tr>
<td>ROR-γt-R</td>
<td>5′-CCAGGAGTAGGCCACATTACA-3′</td>
<td>21</td>
<td>61.3</td>
<td>52.38</td>
</tr>
<tr>
<td>GAPDH-F</td>
<td>5′-CAGTGCCACCCAAGACTG-3′</td>
<td>20</td>
<td>55.20</td>
<td>60</td>
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<tr>
<td>GAPDH-R</td>
<td>5′-CCAGTGAAGCTTCCGGTGCA-3′</td>
<td>20</td>
<td>56.80</td>
<td>60</td>
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</tbody>
</table>

### Table 2 Evaluation of clinical scores (MMCS, AMCS, and CDI) in treated groups

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>MMCS (mean ± SE)</th>
<th>AMCS (mean ± SE)</th>
<th>CDI (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATRA-treated EAE</td>
<td>1.16 ± 0.40</td>
<td>0.76 ± 0.12†</td>
<td>6.91 ± 2.53</td>
</tr>
<tr>
<td>Calcitriol-treated EAE</td>
<td>0.42 ± 0.13*</td>
<td>0.21 ± 0.03**</td>
<td>2.85 ± 1.03*</td>
</tr>
<tr>
<td>Combined-treated EAE</td>
<td>0.68 ± 0.13*</td>
<td>0.33 ± 0.05**</td>
<td>4.43 ± 1.07*</td>
</tr>
<tr>
<td>Vehicle-treated EAE</td>
<td>1.68 ± 0.38</td>
<td>1.07 ± 0.15</td>
<td>12.50 ± 2.55</td>
</tr>
</tbody>
</table>

MMCS, AMCS, and CDI were significantly attenuated in combined (at suboptimal doses) and calcitriol (at optimal doses) groups compared to vehicle-treated group. (†P < 0.05, **P < 0.001 versus vehicle-treated EAE group; *P < 0.05 versus combined- treated EAE group).
immune responses. FOXP3 is the most specific hallmark of Treg cell subsets. FOXP3 expression and stability are closely related to the functionality of Treg cells.\textsuperscript{57} The study of molecular mechanisms demonstrated that ATRA markedly increases the activation of the ERK1/2 signaling pathway, and the resultant signaling promotes FOXP3 expression.\textsuperscript{58} ATRA enhances the differentiation and stability of iTreg cells via increased histone methylation and acetylation within the promoter and conserved noncoding DNA sequence elements at the FOXP3 gene locus.\textsuperscript{58} ATRA can also inhibit methylation of Foxp3 gene of nTreg cells in the presence of inflammatory cytokines.\textsuperscript{59} However, in our study, compared with the vehicle group, the FOXP3 and TGF-\(\beta\) mRNA levels in ATRA-treated group were not significantly changed. In accordance with our results, some studies have found that there is no induction of regulatory T cells when treating inflammatory diseases with RAR agonists,\textsuperscript{42,54} and it has been speculated that this may be due to a lack of TGF-\(\beta\) \textit{in vivo}. An alternative hypothesis is that FOXP3+ T-cell generation is inhibited by the strong induction of inflammatory cytokines, including IL-6, TNF-\(\alpha\), and IL-1.\textsuperscript{54} On the other hand, ATRA administration increased FOXP3 expression and decreased IL-17 expression in colon tissues and mesenteric lymph node cells of BALB/C mice with autoimmune colitis.\textsuperscript{60} Similar treatment with ATRA in diabetic NOD mice upregulated differentiation of Treg cells in splenocytes without any change in Th17 population.\textsuperscript{51} In the

![Figure 1](image1.png) Clinical scores for different treatment groups, including ATRA, calcitriol, combined (ATRA + calcitriol) and vehicle recorded daily during treatment. Preventive treatment by vitamins led to significant reduction of clinical disability from day 13. Data were shown as mean clinical score \(\pm\) S.E.M. for eight mice tested daily in each group \((P < 0.05)\).

![Figure 2](image2.png) Expression of the FOXP3 mRNA levels in the splenocytes of treatment groups determined by real-time PCR. The values were presented as mean \(\pm\) S.E.M. of eight mice in each group.\textsuperscript{*}P < 0.05 versus vehicle-treated group. \textsuperscript{+}P < 0.05 versus ATRA-treated group.

![Figure 3](image3.png) Quantitative analysis of the TGF-\(\beta\) mRNA levels in splenocytes of treatment groups determined by real-time PCR. The values were presented as mean \(\pm\) S.E.M. The relative level of TGF-\(\beta\) mRNA was lower in the vehicle group than in the other groups.\textsuperscript{*}P < 0.05 versus vehicle-treated group. \textsuperscript{+}P < 0.05 versus ATRA-treated group.
present report, FOXP3 and TGF-β gene expression were significantly increased in combination but not in ATRA alone treatment. We propose that the minor expression of FOXP3 by individual therapy in vivo may be due to the profound production of inflammatory cytokines (such as IL-6) or conversely low level production of TGF-β in cytokinic milieu induced by MOG35-55 and CFA immunization. However, combination treatment has synergistically more anti-inflammatory effect, therefore promotes expression of FOXP3 and TGF-β mRNA levels. New animal model study suggests that calcitriol may be a positive regulator of the murine FOXP3,41 and Ikzf2 gene encoding Helios,39 a transcription factor that binds to the FOXP3 promoter and stimulates its transcription.62 New data complemented the rodent data and revealed that calcitriol increased human FOXP3 gene expression by a transcriptional mechanism.63 The human FOXP3 and murine FOXP3 genes share homology in a conserved noncoding sequence (+1714 to +2554 relative to the FOXP3 transcriptional start site) that works as an enhancer.64 Within this enhancer region, investigators identified three VDR elements that enhanced promoter activity in a calcitriol-dependent manner.63

**Conclusion**

Our study demonstrated that preventive treatment with combination of half doses of calcitriol and ATRA provides better outcomes compared to individually administered full doses in EAE-induced female mice. In this report, we found the in vivo inhibitory effects of combination treatment of ATRA and calcitriol on the mRNA expression of IL-17, ROR-γt, and stimulatory effects on the mRNA expression of TGF-β and FOXP3 in splenocytes from EAE female mice. The results of this study can be generalized to female mice and to understand the impact of such interventions on male mice, further studies need to be done. In this study, the doses were selected based on previous studies. However, studies on the higher or lower doses than were used in the present study are necessary to achieve more assertive results. Perhaps if in the present study, the doses of calcitriol and ATRA alone and in combination were equal, some results could be better interpreted. However, this survey is a preliminary study. Other mechanisms may also be involved in the synergistic benefits of this combination, and these remain to be clarified. In conclusion, this nutraceutical approach may be promising for the treatment and/or prevention of MS and perhaps other inflammatory demyelination diseases, such as Devic’s disease, chronic relapsing inflammatory optic neuritis, ADEM, and Balo concentric sclerosis.

**Disclaimer statements**

**Contributors** None.

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**Conflicts of interest** The authors declare that there is no conflict of interest.

**Ethics approval** None.
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