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# Immunomodulatory Effect of Calcitriol on Experimental Autoimmune Encephalomyelitis Mice, A Multiple Sclerosis Animal Model

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## ABSTRACT

Previous studies noted an imbalance in T helper (Th) 17 and regulatory T cells (Tregs) in experimental autoimmune encephalomyelitis (EAE), a multiple sclerosis animal model. calcitriol, vitamin D's active form, was found to ameliorate EAE symptoms by favoring Tregss over Th17 cells, suggesting immunomodulatory effects. This study aimed to assess calcitriol's impact on EAE manifestations and cytokine profile in mice.

In this study, we recruited twenty-eight C57BL/6 mice and divided them into 4 groups: healthy controls, EAE, EAE with calcitriol treatment, and healthy mice with calcitriol treatment. $CD4^+$ T cells were isolated from splenocytes using magnetic-activated cell sorting. Real-time polymerase chain reaction was employed to quantify the genes associated with Th9 cells (i.e., *SPI1* encoding PU.1 and *IL9* encoding interleukin [IL]-9). Moreover, the levels of IL-17 and transforming growth factor beta (TGF-β) were evaluated through enzyme-linked immunosorbent assay in the supernatant of $CD4^+$ T cell culture stimulated by anti-CD3 and anti-CD28 antibodies for 72 hours.

In the supernatant of $CD4^+$ T cell cultures, the levels of interleukin-17 (IL-17) were significantly increased, while the levels of transforming growth factor beta (TGF-β) were decreased in the EAE Group compared to the healthy control group. Calcitriol treatment reversed these changes and attenuated EAE symptoms, as confirmed in hematoxylin and eosin, and luxol fast blue stains. Notably, calcitriol increased *IL9* gene expression in both EAE and healthy mice.

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This study provides further evidence of the anti-inflammatory effects of calcitriol and its role in attenuating EAE.

**Keywords:** Calcitriol; Experimental autoimmune encephalomyelitis; *IL-9*; IL-17; Transforming growth factor beta; Th9 Cells

## INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) characterized by the degradation of myelin and neural cell destruction. Immunological, genetic, and histopathological investigations of MS patients have strongly indicated that autoimmunity plays an important role in the disease's pathogenesis. The disease involves the infiltration of inflammatory cells into CNS areas, resulting in attacks on the myelin sheath, leading to inflammation and subsequent damage to myelin and axons, ultimately causing CNS impairment.[1]

To study MS, scientists often employ experimental autoimmune encephalomyelitis (EAE) as an animal model, as it exhibits many key aspects of the disease. Most insights into MS have been derived from studies on animal models, particularly EAE, which currently stands as the most reliable approach for MS research. EAE can be induced in various species, but mice are predominantly used due to their ability to generate genetically diverse mice strains.[2] EAE is an autoimmune disease primarily mediated by T-helper (Th) cells, characterized by the infiltration of T cells and monocytes in the CNS, leading to localized inflammation. Autoimmunity targets proteins expressed by myelin-producing oligodendrocytes in the CNS, resulting in early demyelination of axonal pathways, impaired CNS axonal conduction, and progressive hindlimb paralysis.[3]

Vitamin D plays specific roles in the immune response, derived mainly from progenitor cells in different tissues and body cells, functioning through nuclear receptor binding. Numerous nucleated cells in the body, including oligodendrocytes, neurons, microglia, and astrocytes, express the vitamin D receptor (VDR) and respond directly to vitamin D. Immune system cells such as macrophages and active T cells also express VDR, signifying the vitamin's role in immune function. The active form of vitamin D, 1,25-dihydroxyvitamin D, acts as a hormone regulating the immune system, reducing the number and activity of pro-inflammatory Th1 and Th17 cells, showing positive effects in autoimmune diseases.[4]

Th17 cells are characterized by their production of the cytokine interleukin (IL)-17 and the transcription factor *RORγt*. These cells secrete various cytokines, including IL-17A, IL-17F, IL-6, IL-9, IL-21, IL-22, IL-23, IL-26, Granulocyte-macrophage colony-stimulating factor (GM-CSF) and Tumor necrosis factor-α (TNF-α). IL-17A, being a specific cytokine of Th17 cells, induces the production of other pro-inflammatory cytokines and soluble mediators, including IL-6, IL-1, TNF-α, GM-CSF, matrix metalloproteinase (MMP) and CXCL8 in various cells, exacerbating inflammation. Studies have demonstrated an increase in *IL17* gene expression in the spinal cord and the serum level of this cytokine in mice with EAE. Additionally, researchers have found that the deletion of the *IL17* gene in EAE revealed its greater significance in disease pathogenesis compared to IFN-γ.[5,6]

Regulatory T cells (Tregs) play a critical protective role in EAE by producing cytokines such as transforming growth factor beta (TGF-β) and IL-10. Factors that effectively shift the immune system from Th1 to Th2 and from Th17 to Treg could potentially aid in treating or preventing the progression of MS.[7] Research has demonstrated that priming naive T cells in the presence of TGF-β along with IL-4 induces their differentiation towards Th9 cells, known for producing IL-9 cytokines. Although varying amounts of IL-9 are produced by Th2 and Treg cells under different conditions and diseases, Th9 cells are known as specialized IL-9-producing cells.[8] In general, IL-9 production by T cells appears to contribute to immunosuppression. The identification of Th9 cells has led to the discovery of cytokines that either enhance or inhibit their differentiation.[9] The transcription factor PU.1 has been found to be essential for Th9 development, as T cells with defects in the PU.1 coding gene are unable to differentiate into Th9 cells, which subsequently reduces airway inflammation.[10] The objective of this study was to explore the impact of calcitriol on the symptoms in EAE mice. Considering


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the significant roles played by Th17 and Treg cells in the inflammatory and anti-inflammatory processes during EAE pathogenesis, our study aimed to investigate the anti-inflammatory properties of calcitriol on the inflammatory cytokine IL-17, the anti-inflammatory factor TGF-β and the expression of anti-inflammatory genes *IL-9,* PU.1 in EAE mice.

## MATERIALS AND METHODS

### Animals

A total of 28 female C57BL/6 mice, aged between 6 and 8 weeks, were procured from Pasteur Institute of Iran, Karaj, Iran. The mice were housed in a controlled environment with a 12-hour light-dark cycle and maintained at a temperature ranging from 20 to 25°C. They were provided with ad libitum access to food, adhering to the principles of laboratory animal care. The experimental procedures took place at the School of Public Health, Tehran University of Medical Sciences, and were conducted following the guidelines and regulations set forth by the Ethics Committee, approved under the code number IR.TUMS.SPH.REC.1396.3158.

### Group and Study Design

The animals were divided into 4 groups, each comprising 8 mice. The groups were designated as follows:

Group C (healthy control group), Group E (EAE without intervention), Group E + Vit D (EAE group + intraperitoneal (IP) injection of calcitriol), Group C + Vit D (healthy control group + IP injection of calcitriol)

In the control groups, the solvent for the calcitriol drug was employed as a vehicle in this study.

### EAE Induction and Treatment of Mice

The induction of the EAE mouse model followed the guidelines provided in the Hooke company kit (Hooke laboratories, EK-2110, Lawrence, MA, USA). Briefly, each mouse in both the EAE Group and the intervention Group received a subcutaneous injection of 200 μL of myelin oligodendrocyte glycoprotein (MOG) emulsion combined with complete Freund's adjuvant (CFA) in both the left and right flanks (100 mL each flank). In the intervention group, IP injections of calcitriol commenced one day prior to EAE induction and were administered until day 21. Each mouse in the intervention group received 100 ng of calcitriol via IP injection every other day. Similarly, the healthy control group received the same amount of calcitriol as the intervention group. Mice from all groups were euthanized using anesthesia on day 21 post-induction. Initially, the mice were anesthetized via IP injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). A symptom-based scoring method was employed.

The severity of symptoms in mice was assessed daily based on the following scale: healthy (0), partial tail paralysis (0.5), complete tail paralysis (1), complete tail paralysis and relative hind leg weakness (1.5), complete tail paralysis and complete hind leg weakness (2), paralysis of one hind leg (2.5), complete hind leg paralysis (3), leg paralysis and front leg weakness (3.5), complete paralysis of all four legs (4), and ultimately, death (5).

### Isolation of $CD4^+$ T Cells from Mouse Splenocytes

The isolation of $CD4^+$ T cells from mouse splenocytes involved several precise steps. Firstly, the spleen was extracted from the mice, and its tissue was gently crushed using the bottom of a sterile syringe plunger in a petri dish containing 5% fetal bovine serum in RPMI 1640 medium (Gibco, USA), which served as the culture medium. Subsequently, the crushed spleen tissue was passed through a blunt 23-gauge needle multiple times to obtain single cells. These cells were then transferred into a 15 mL Falcon tube and washed by centrifugation at 500*g* for 10 minutes at room temperature, after which the supernatant was discarded. To remove splenic red blood cells (RBCs), we utilized RBC lysing buffer. Specifically, we employed ammonium-chloride-potassium (RBC lysis buffer, Sigma-Aldrich, USA) for 3 minutes and washed the cells by centrifuging at 300*g* for 10 minutes at room temperature. The next step involved the extraction of $CD4^+$ T cells using the magnetically activated cell separation (MACS) technique (Miltenyi, Germany). For this purpose, after determining the concentration of the cell suspension and counting the cells to obtain the required amount of $CD4^+$ T cells for subsequent tests, we suspended a specific number of spleen cells in a specific volume of MACS buffer. Then, the spleen cells, excluding $CD4^+$ T cells, were labeled with biotinylated antibodies against the surface markers present on these cells. After incubation at 4°C for 5 minutes, magnetic nanoparticles conjugated to primary antibodies against biotin were added to the cell suspension, and the cells were incubated for 10 minutes at 4°C. Finally, based on the abundance of $CD4^+$ T cells in the spleen, we employed the appropriate column to extract $CD4^+$ T cells.


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### Quantitative Gene Expression Assessment

Total RNA was isolated from $2.5\times10^6$ $CD4^+$ T cells using an RNA extraction kit (Biobasic, Canada) following the manufacturer's instructions. The extracted RNA purity was determined by assessing absorbance ratios at 260/280 and 260/230. Additionally, denaturing gel electrophoresis (1%) was employed, and the density of rRNA bands (28S and 18S) was observed to assess RNA integrity). For cDNA synthesis, the TAKARA Primer Script™ RT reagent Kit (Japan) was used to convert purified RNA to cDNA. Gene expression levels of *IL9* and *SPI1* (encoding PU.1); encoding were quantified using the ABI StepOnePlus real-time quantitative polymerase chain reaction (RT-qPCR) System (Applied Biosystems, USA) with SYBR Green PCR Master Mix (Takara, Japan). The mean threshold cycle (Ct) was recorded for each sample, and β-actin was chosen as the housekeeping gene. The expression of *IL9* and *SPI1* mRNA relative to β-actin mRNA was measured using the $2^{-\Delta\Delta Ct}$ method, providing a reliable quantitative analysis. Detailed information regarding all primers used in the RT-qPCR can be found in Supplementary Table.

### Culture and Stimulation of $CD4^+$ T Cells for ELISA

To assess the cytokine production by $CD4^+$ T cells, the cells were stimulated using antibodies against CD3 and CD28 (Ebioscience,.USA). The supernatant from the cell culture was then collected to investigate the levels of IL-17 and TGF-β1 ((Ebioscience,.USA) cytokines. For this purpose, $CD4^+$ T cells were seeded on a 48-well plate at a concentration of $5\times10^5$ cells per well. The cells were stimulated under the following conditions: 0.5 µg/mL of anti-CD3 and 0.2 µg/mL of anti-CD28. The cells were allowed to culture for 72 hours. On the third day, the culture supernatant was collected for the enzyme-linked immunosorbent assay (ELISA). This assay aimed to quantify the levels of IL-17 and TGF-β1 cytokines produced by the stimulated $CD4^+$ T cells during the culture period.

### Histopathology

On the 21st day after EAE induction, we isolated the spinal cord from mice and fixed it in formalin. The fixed spinal cord was then embedded in paraffin for further processing. Subsequently, the lumbar part of the spinal cord was carefully sectioned into 5 to 7 µm-thick slices for histological evaluation. To investigate the presence of inflammatory cell accumulation in the nervous system, we employed the hematoxylin and eosin (H&E) staining method. This staining allowed us to visualize and assess the extent of inflammation in the spinal cord tissues. Furthermore, to evaluate the demyelination of nerve cells, we utilized the luxal fast blue (LFB) staining method. This staining technique is specifically designed to highlight the myelin sheaths of nerve fibers, enabling us to identify and quantify any demyelination occurring in the spinal cord.

### Statistical Evaluation

The variable values in each group were expressed as mean±standard deviation. A significance level of $p<0.05$ was considered significant for all tests. The Prism 8.0 software was employed for data analysis, and the same software was used for generating graphs. Initially, the Kolmogorov-Smirnov test assessed the normality of data distribution. If the data were normally distributed, parametric tests were applied; otherwise, nonparametric tests were utilized.

For the analysis of multiple groups, parametric tests included the one-way analysis of variance (ANOVA) followed by Tukey's post hoc test to identify differences between groups. Nonparametric tests, on the other hand, encompassed the Kruskal-Wallis test followed by Dunn's post hoc test to assess differences between groups.

In cases involving the comparison of two groups, parametric tests included the independent-samples *t*-test, while nonparametric tests involved the Mann-Whitney test.

## RESULTS

### The Effect of Calcitriol on the Severity of Clinical Symptoms

In Group E, clinical symptoms appeared on day 12 after EAE induction in mice, with a prevalence rate of 100% by the end of the test period. The severity score of clinical symptoms for each EAE mouse gradually increased until day 21, reaching a range between 2 and 3.5 on that day, at which point the mice were sacrificed. In Group E + Vit D, the clinical symptoms in mice commenced on day 16, and by day 21, the severity of clinical symptoms ranged from 0.5 to 1, with a prevalence rate of 75% by the end of the test period. Figure 1A illustrates a significant difference in disease prevalence between Group E and Group E + Vit D ($p<0.05$). The severity score of clinical symptoms in both groups is


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depicted in Figure 1B, demonstrating a significant difference between the two groups *($p<0.01$)*.

## Effect of Calcitriol on Weight

The weight of the mice was diligently recorded daily throughout the study, and a weight monitoring chart was constructed to track changes over the study period. In Group C, the mice exhibited relatively stable or insignificantly increasing weights over time (Figure 2A). In Group E, a noticeable weight loss was observed from the onset of clinical symptoms (Figure 2B). Surprisingly, in Group E + Vit D, weight loss in mice commenced even before the appearance of clinical symptoms (Figure 3A). In Group C + Vit D, the weight of the mice began to decrease on the 12th day (Figure 3B).

A compelling comparison between the weight trends of Groups C and E demonstrated a significant difference, with the weight of mice in Group C being higher than that of mice in Group E on day 21 ($p<0.01$) (Figure 4A). Furthermore, a comparison between the weight trends of Groups E and E + Vit D indicated a significant decrease in the weight of mice in the E + Vit D Group compared to the E Group on day 21 ($p<0.0001$) (Figure 4B).

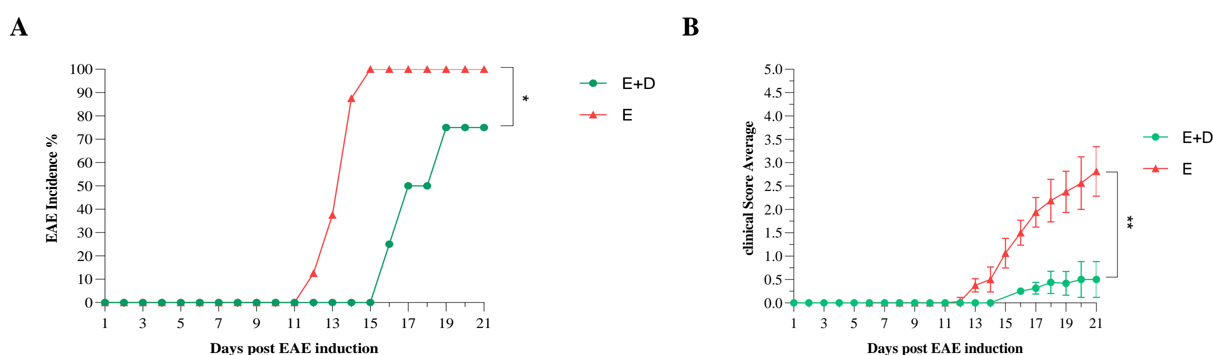


**Figure 1. Experimental Autoimmune Encephalomyelitis (EAE) Incidence and Clinical score average in the studied groups. (A) In Group E and Group E + Vit D, the prevalence rate was 100% and 75%, respectively, at the end of the test period. Disease prevalence was significantly increased in Group E compared to Group E + Vit D (*$p$<0.05). (B) The onset of disease symptoms in Group E occurred on the 12th day, whereas in Group E + Vit D, the disease symptoms began on the 16th day. The clinical score average was significantly increased in Group E compared to Group E + Vit D. The statistical analysis was performed using Mann-Whitney, and significance levels are represented as follows: *$p$<0.05, **$p$<0.01**

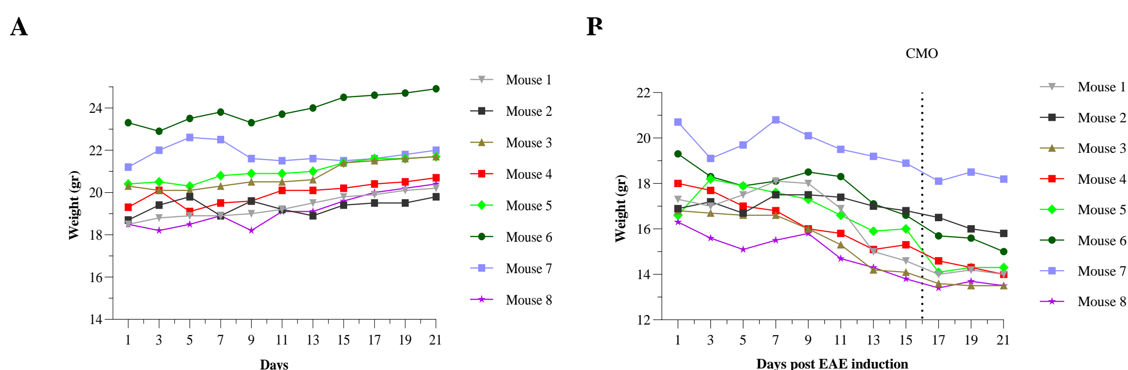


**Figure 2. Weight monitoring chart of Group C and Group E + Vit D mice during the study period. (A) The weight monitoring chart illustrates the weight trends of mice in the control group (Group C) over the entire study period. Remarkably, the mice in the control group exhibited minimal or negligible changes in their weight throughout the study. In some instances, slight weight increments were observed. Each line graph represents an individual mouse in this group, with each point representing a recorded weight on a specific day. (B) The weight of mice in Group E + Vit D started to decrease even before the onset of clinical symptoms. The clinical manifestation of symptoms was observed to commence on day 16, as indicated by the dotted line. Each line graph represents an individual mouse in this group, and each data point represents the recorded weight on a specific day. CMO: clinical manifestation onset.**


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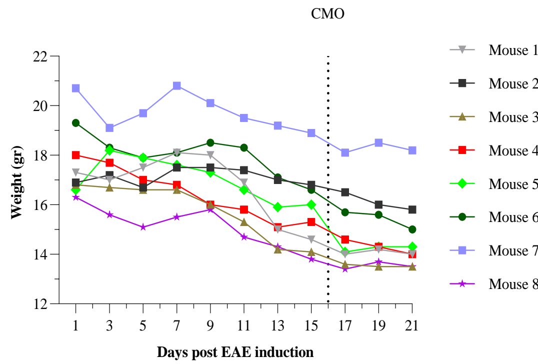
A

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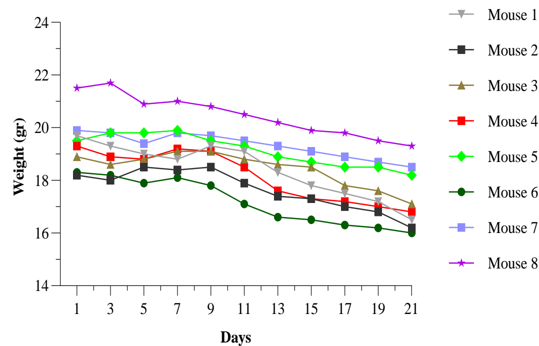
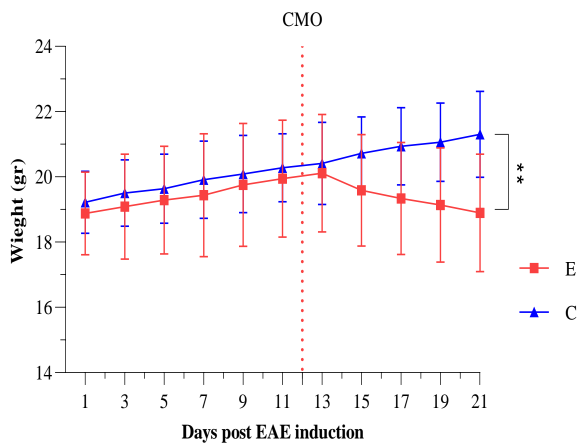



**Figure 3. Weight monitoring chart of mice in Group E + Vit D and Group C + Vit D during the study period. (A) The weight monitoring chart of the mice in Group E + Vit D clearly illustrates a notable decline in weight before the onset of clinical symptoms. Clinical symptoms began to manifest on day 16, as indicated by the dotted line. Each line graph in the figure corresponds to an individual mouse in this group, and each data point represents the recorded weight on a specific day. (B) The weight monitoring chart of mice in Group C + Vit D clearly shows evident weight loss during the study period. Each line graph in the figure corresponds to an individual mouse in this group, and each data point represents the recorded weight on a specific day. CMO: clinical manifestation onset.**



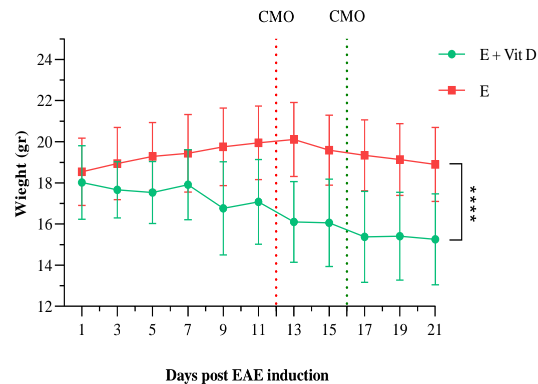




**Figure 4. Weight comparison between Groups E and E + Vit D and Groups E and E + Vit D during the study period. (A) The weight comparison graph between mice in Groups E and C clearly illustrates that with the onset of clinical symptoms in mice of Group E, their weight decreased, while the weight of mice in Group C remained comparatively higher over time. The statistical comparison between the two groups using an unpaired *t*-test revealed a significant difference, with mice in Group C exhibiting greater weight than mice in Group E throughout the study (*p<0.01*). The dotted line indicates the time of onset of clinical symptoms. Values are presented as mean± standard error. The statistical analysis was performed using an unpaired t-test, and significance levels were represented as follows: ***p<0.01*; CMO: clinical manifestation onset. (B) Group E mice began to experience weight loss on day 12, concurrent with the onset of clinical symptoms. In contrast, the mice in Group E + Vit D exhibited a delayed onset of clinical symptoms compared to Group E. The green dotted line indicates the onset time of clinical symptoms in the E + Vit D group, while the red dotted line indicates the onset time of clinical symptoms in the E group. Statistical analysis, employing an unpaired t-test, demonstrated a remarkable and highly significant difference in weight between the two groups (*****p*<0.0001). Values are presented as mean ± standard error. The statistical analysis was performed using an unpaired t-test, and significance levels were represented as follows: *****p<0.0001*.**

**Investigation of *IL9* and *SPI1* Gene Expression in Relation to Th9**

We conducted a quantitative analysis of the expression of *IL9* and *SPI1* genes associated with the Th9 subtype. This investigation involved RNA extraction from $CD4^+$ T cells, which were purified from splenocyte cells of mice in the C, E, E + Vit D, and C + Vit D groups. Upon treatment with vitamin D, there was a significant increase in the expression of *IL9* in the E + Vit D Group when compared to the E Group *($p<0.0001$)*. Additionally, vitamin D administration resulted in an elevated expression level of *IL9* in the C + Vit D Group in comparison to the C Group *($p<0.01$)*. Interestingly, the induction of EAE and vitamin D treatment did not induce significant changes in the expression of transcription factor PU.1. These findings, as depicted in Figures 5A and 5B, suggest that vitamin D has a modulatory effect on *IL9* gene expression related to Th9 cells, but does not exert a similar impact on the PU.1 transcription factor expression.

**Investigation of IL-17 and TGF-β1 Cytokine Levels in $CD4^+$ T Cell Culture Broth**

To assess the production levels of IL-17 and TGF-β1 cytokines by $CD4^+$ T cells in Groups C, E, and E + Vit D, these cells were stimulated with antibodies against CD3 and CD28 for 72 hours. The culture supernatant was subsequently collected to quantify the amount of these cytokines produced. Upon analysis, the concentration of IL-17 in the culture broth of $CD4^+$ T cells from Group E exhibited a significant increase compared to Groups C *($p<0.0001$)* and E + Vit D *($p<0.05$)*. In contrast, the secretion of TGF-β1 cytokine by $CD4^+$ T cells in Group E showed a notable and significant decrease when compared to Groups C *($p<0.001$)* and E + Vit D *($p<0.05$)* (Figures 6A and 6B).

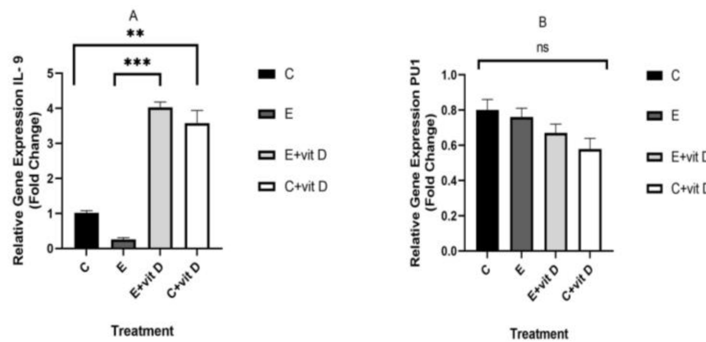



**Figure 5. Examination of interleukin (IL)-9 and *SPI1* gene (coding PU.1) expression in $CD4^+$ T cells of the studied groups. (A) A remarkable and significant increase in *IL9* gene expression was observed in the E + Vit D Group compared to the E Group. Similarly, the C + Vit D Group exhibited a higher expression of *IL9* compared to the C Group. (B) Intriguingly, the induction of Experimental Autoimmune Encephalomyelitis (EAE) and treatment with vitamin D did not result in significant changes in the expression of transcription factor PU.1. The statistical analysis was performed using one-way analysis of variance (ANOVA). *$p<0.05$, **$p<0.01$, and ***$p<0.001$.**

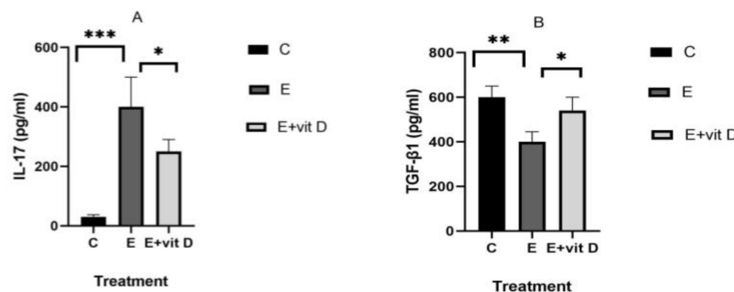



**Figure 6. Analysis of IL-17 and TGF-β1 cytokine production in the $CD4^+$ T cell culture supernatant of the studied groups. (A) IL-17 cytokine production was significantly increased in Group E compared to Group C ($p<0.0001$) and decreased in Group E + Vit D compared to Group E. (B) Regarding TGF-β1 cytokine production, Group E exhibited a significant reduction compared to both Group C and Group E + Vit D. The statistical analysis was performed using one-way ANOVA. *$p<0.05$, **$p<0.01$, and ***$p<0.0001$.**



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## Accumulation of Inflammatory Cells in the Spinal Cord

On the 21st day after EAE induction, the spinal cord of mice was isolated and preserved through formalin fixation and paraffin embedding. Subsequently, the accumulation of inflammatory cells in the spinal cord tissue was assessed using H&E staining. In Group E, evident accumulation of inflammatory cells was observed in the spinal cord tissue (Figure 7A). However, in Groups C (Figure 7B) and E + Vit D (Figure 7C), there was no observable accumulation of inflammatory cells in the spinal cord tissue.

## Examination of Spinal Cord Demyelination by Luxol Fast Blue Staining

On the 21st day after the induction of EAE, the spinal cords of mice were isolated and preserved through formalin fixation and paraffin embedding. Subsequently, the demyelination of inflammatory cells in the spinal cord tissue was assessed using LFB staining, a myelin-specific staining technique. In Group E, demyelination was evident in the spinal cord tissue (Figure 8A), clearly indicating a loss of myelin. However, in Groups C (Figure 8B) and E + Vit D (Figure 8C), no demyelination was observed, signifying the preservation of myelin integrity.

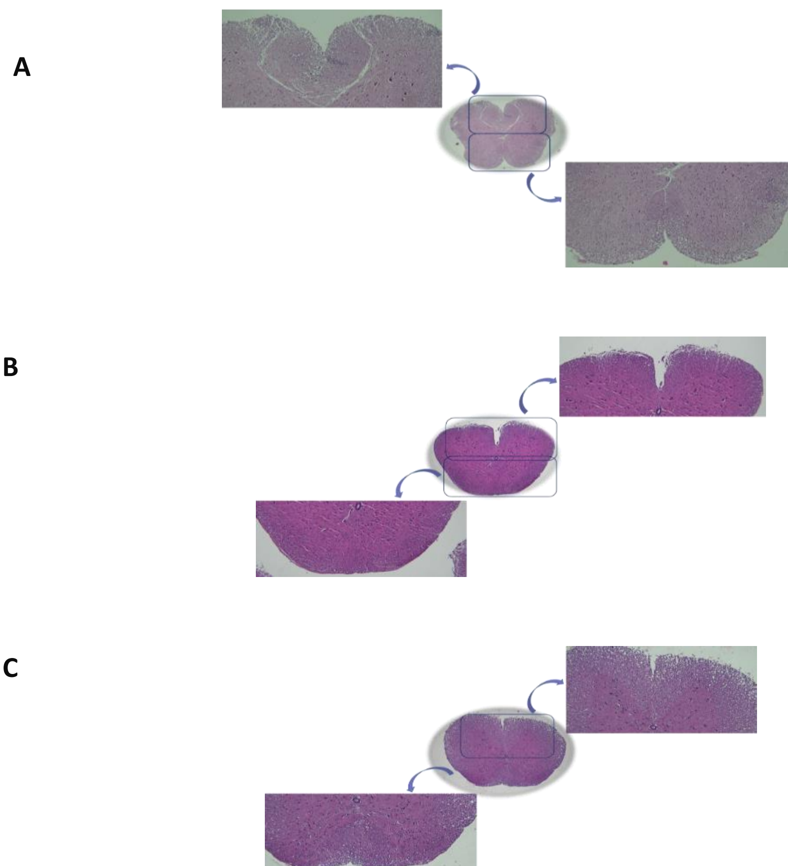


**Figure 7. Hematoxylin and eosin (H&E) staining in spinal cord tissue of the mice. (A) H&E staining of the spinal cord tissue from Experimental Autoimmune Encephalomyelitis (EAE) mice reveals the presence of inflammatory cell accumulation, depicted by the arrows. The middle figure is magnified at 40x, while the other two figures are magnified at 100x. (B) H&E staining of the spinal cord tissue from healthy mice shows no evidence of inflammatory cell accumulation. The absence of inflammatory cells is evident in the figures, with the middle figure magnified at 40x and the other 2 figures magnified at 100x. (C) H&E staining of the spinal cord tissue from EAE mice treated with vitamin D reveals no evidence of inflammatory cell accumulation. The absence of inflammatory cells is clearly observed in the figures, with the middle figure magnified at 40x and the other 2 figures magnified at 100x.**


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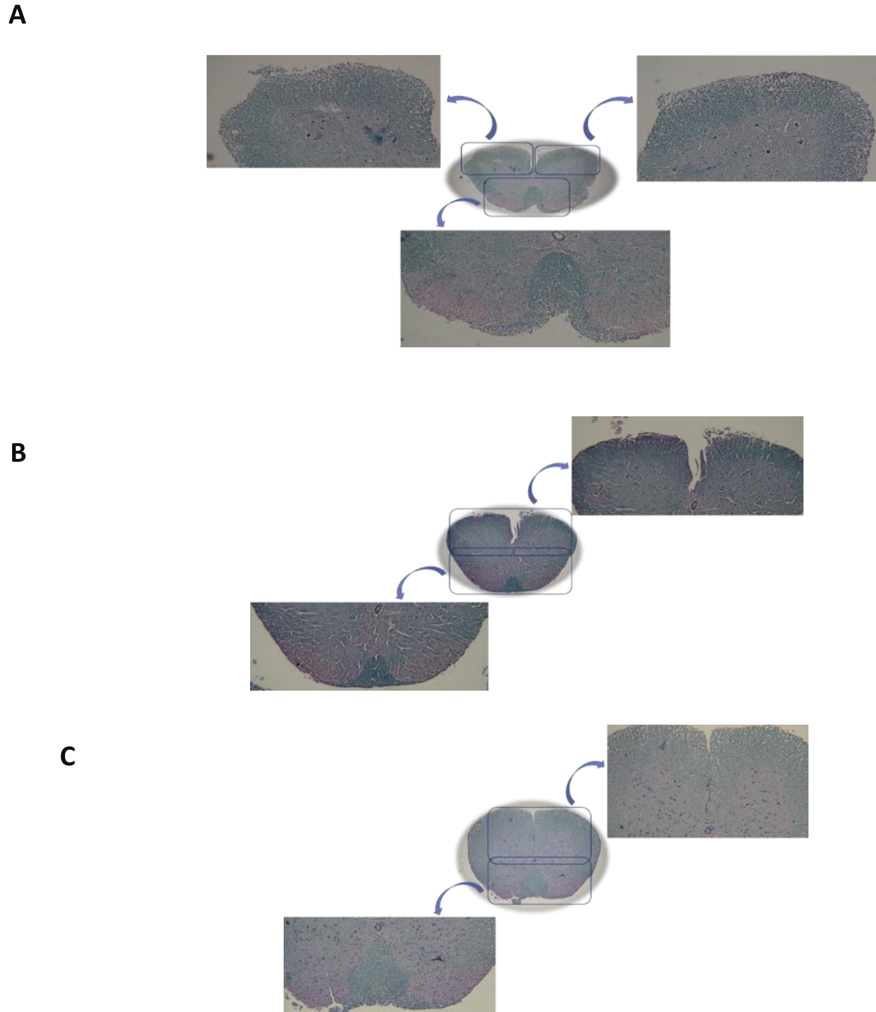


**Figure 8. Luxol Fast Blue Staining (LFB) staining in spinal cord tissue of mice. (A) LFB staining of the spinal cord tissue from EAE mice reveals an area with demyelination, clearly indicated by the presence of demyelination with the middle figure magnified at 40x, and the other 3 figures magnified at 100x. (B) LFB staining of the spinal cord tissue from healthy mice shows no trace of demyelination. The absence of demyelination is clearly evident in the figures, with the middle figure magnified at 40x, and the other 2 figures magnified at 100x. (C) LFB staining of the spinal cord tissue from EAE mice treated with vitamin D shows no trace of demyelination. The absence of demyelination is clearly observed in the figures, with the middle figure magnified at 40x, and the other 2 figures magnified at 100x.**

## DISCUSSION

This study aimed to investigate the effect of the active form of vitamin D (calcitriol) on the expression of the *IL9* gene and the transcription factor PU.1, related to Th9 cells, as well as the secretion of anti-inflammatory (TGF-β1) and inflammatory (IL-17) cytokines in $CD4^+$ T cells isolated from mice with EAE, compared to healthy mice. Consistent with previous research, our findings demonstrated that the EAE Group treated with vitamin D (Group E + Vit D) experienced a delayed disease onset (day 16 vs. day 12) compared to the untreated group (Group E). Moreover, the mean clinical score in Group E + Vit D was significantly lower compared to the EAE group, indicating a reduction in inflammation in the CNS of mice. These results are in line with existing research on the impact of calcitriol on the severity of clinical symptoms in the EAE model.[11]

Assessment of the accumulation of inflammatory cells and demyelination in the spinal cord of EAE mice through H&E and LFB staining revealed these lesions were present only in EAE mice and not in Group E + Vit D. These findings are consistent with previous studies investigating the effect of different doses of calcitriol on


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the inflammatory cell accumulation and demyelination in the spinal cord.[11]

Additionally, our study observed weight loss in the EAE group compared to the healthy control group, as well as weight loss in the groups receiving calcitriol, compared to their respective control groups (Group E + Vit D compared to Group E and also Group C + Vit D compared to Group C). Weight loss in EAE mice is a characteristic clinical sign, and its severity correlates with disease progression. It can be attributed to factors such as paralysis, limited access to food, increased production of inflammatory cytokines like TNF-α during the acute inflammatory phase, inability to chew, and potential digestive problems. Notably, if there is no treatment in EAE mice, after passing the peak of the disease, weight gain begins, so that on the 28th day after EAE induction, the mice have about 90% of their initial weight. In our study, according to previous studies, mice were euthanized on the 21st day after induction, which is the peak of the disease and clinical symptoms and, as a result, the maximum weight loss. Weight loss of mice as a result of EAE induction has also been observed in other studies by our research group and others.[12,13]

As mentioned, in our study, weight loss was observed in groups receiving calcitriol compared to control groups. Many studies have been conducted regarding the role of vitamin D in the regulation of fat and sugar metabolism.[14,15] Research in the field of overweight and obesity has shown the relationship between serum vitamin D levels and body mass index (BMI), fat mass, hyperinsulinemia, insulin resistance, and inflammatory markers such as C-reactive protein.[16]

Circulating levels of vitamin D in humans have consistently shown an inverse correlation with fat mass, with numerous studies indicating lower vitamin D levels in obese individuals and an inverse relationship with BMI.[16] This points to the critical role of calcitriol in adipocyte metabolism by inhibiting adipogenesis.[17] By blocking the differentiation of adipocyte progenitor cells into mature adipocytes, vitamin D effectively reduces fat production.[15] Animal models further support the protective role of vitamin D in diet-dependent obesity, where it enhances fatty acid oxidation and promotes adipose tissue apoptosis.[18] Research by Wenclewska et al, has demonstrated that calcitriol increases intracellular calcium levels, leading to adipocyte apoptosis.[19] Supplementation with vitamin D along with calcium has been shown to reduce fat accumulation by boosting adipocyte apoptosis.[20] Additionally, calcitriol directly influences leptin gene expression, a satiety hormone, and its secretion. Vitamin D also appears to influence insulin secretion and sensitivity, with low vitamin D levels being a predictive factor for type 2 diabetes.[21] Studies suggest that vitamin D deficiency contributes to insulin resistance, leading to metabolic disorders such as hyperglycemia and dyslipidemia. Notably, Wenclewska et al, demonstrated that a daily intake of 2000 IU of vitamin D for 3 months reduced parameters associated with insulin resistance and improved glucose and fat metabolism in a study conducted in 2019.[19] Furthermore, the effect of vitamin D on reducing insulin resistance has also been observed in mice.[22] Vitamin D deficiency in mice results in impaired glucose and insulin homeostasis, and supplement therapy improves these conditions.[23] Reduced insulin resistance subsequently leads to decreased appetite and increased satiety, contributing to effective weight loss.[24] In summary, scientific evidence supports the notion that vitamin D exerts various effects on sugar and fat metabolism through different mechanisms. These effects include inhibiting the production and differentiation of fat cells, promoting adipocyte apoptosis, increasing insulin sensitivity, and subsequently reducing appetite. These confirmed effects of vitamin D may account for the weight loss observed in the calcitriol-receiving groups in our study. As expected, the weight loss in the groups E and E+D is more pronounced than in the C and C+D groups due to the synergistic effect of EAE induction and calcitriol treatment. Our study results demonstrate, for the first time, that calcitriol supplementation significantly increases the expression of the *IL9* gene in $CD4^+$ T cells of both EAE and healthy mice. Additionally, although the expression of *IL-9* in EAE mice showed a slight decrease compared to healthy mice, this difference was not statistically significant. Regarding the PU.1 transcription factor, no significant changes were observed in the studied groups. Based on the results, it appears that treatment with calcitriol increased the activity of Th9 cells in terms of IL-9 production, but it had minimal effect on the number and degree of differentiation of these cells. IL-9 is a cytokine originally identified as a Th2 cell cytokine, involved in response against helminth infections, asthma, and chronic obstructive pulmonary disease. Th17 cells, Tregs, and mast cells also produce IL-9, but Th9 cells are the main producers of this cytokine.[25]


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Early studies suggested that IL-9 played a pathogenic role in various inflammatory diseases. However, recent evidence indicates that IL-9 can also limit pathogenic autoimmune responses. For example, adaptive induction of EAE by myelin-specific Th9 cells was shown to produce milder disease compared to Th17 transfer.[26] Additionally, IL-9 can enhance the inhibitory function of Treg, and mice lacking IL-9 receptor (IL-9R) develop more severe EAE compared to normal mice.[(27)] These findings suggest that IL-9 may play a regulatory role in EAE, although the specific mechanisms by which IL-9 inhibits the disease remain unclear. As IL-9 is a cytokine with pleiotropic functions, further research is needed to determine its role in the control of autoimmune inflammation. In a study by Rostami et al, it was demonstrated that the absence of IL-9 or its receptor increased the severity of EAE symptoms, possibly due to increased abundance of GM-CSF$^{+}$ helper T cells and activation of dendritic cells (DCs) in the CNS. They also found that administering IL-9 delayed the progression of the disease by inhibiting the production of GM-CSF by helper T cells and modulating DCs. Their observations further revealed that the adoptive transfer of IL-9R$^{-/-}$ DCs into normal mice resulted in increased disease severity through the abundance of GM-CSF+ helper T cells, while the adoptive transfer of normal DCs into IL-9$^{-/-}$ mice had no effect on the development of EAE. In summary, these researchers concluded that IL-9 controls the severity of EAE by modulating the production of GM-CSF by T cells via DCs.[28] Few studies have been conducted on the role of IL-9 in MS. In one study by Muls et al, in 2012, they showed that in 14 relapsing-remitting multiple sclerosis (RRMS) patients, the ratio of IL-9$^{+}$CD3$^{+}$ cells to IL-17$^{+}$CD3$^{+}$ cells was reduced compared to controls. However, the frequency of IL-9-producing CD3$^{+}$ T cells was not significantly different between the two groups.[(29)] Another study found that IL-9 levels in cerebrospinal fluid (CSF) decreased during relapses and increased following prednisolone treatment. This suggests a role for IL-9 in maintaining the quiescent phase of symptoms.[(30)] In a separate study, researchers showed that CSF IL-9 levels in 107 RRMS patients inversely correlated with inflammatory activity, neurodegeneration, and disability progression in MS. High levels of IL-9 were associated with a lack of IL-17 in the CSF of RRMS patients, indicating a role for IL-9 in immune regulation in these patients.[(31)] These findings support the regulatory role of IL-9 observed in studies conducted in the EAE model. Overall, based on the available evidence and the results of this study, it appears that calcitriol may inhibit inflammation and improve the condition by increasing the expression of IL-9 cytokine from Th9 cells or other cells that produce this cytokine, such as Tregs and Th2 cells. Since very few studies have been conducted on the role of Th9 cells in the mouse model of EAE and MS and the effect of vitamin D on these cells, further research in this field is warranted. It is also suggested that in addition to examining the gene expression related to Th9 cells, protein expression of transcription factors and cytokines associated with them should be evaluated using various techniques, such as western blot and flow cytometry. In the present study, we investigated the levels of IL-17 and TGF-β cytokines using the ELISA method. The secretion rates of these cytokines in the cell culture broth of CD4$^{+}$ T cells isolated from mice were measured 72 hours after stimulation with antibodies against CD28 and CD3. The IL-17 cytokine level in the EAE Group showed a significant increase compared to the healthy control group, while a significant decrease was observed in the EAE + Vit D Group compared to the EAE group. Conversely, the TGF-β cytokine level significantly decreased in the ELISA test for the EAE Group compared to the healthy control group, and it significantly increased in the EAE + Vit D Group. In the study conducted by Haghmorad et al, cytokine measurement was investigated using the ELISA method in the 72-hour culture supernatant of spleen cells and lymph nodes stimulated with MOG35-55 protein (20 μg/mL). In line with gene expression analysis, the prevention and treatment groups with medium and high doses of calcitriol showed decreased levels of inflammatory cytokines (IFN-γ, IL-17, TNF-α, and IL-6) compared to the control group. Moreover, the levels of anti-inflammatory cytokines (IL-10, TGF-β, and IL-4) were increased in these groups.[11] These findings, consistent with our results, confirm the effect of calcitriol in promoting inhibitory cytokine and suppressing inflammatory cytokine. *RORγt* expression is essential for *IL17* expression, and a genetic defect of *RORγt* in mice has been shown to impair Th17 differentiation and protect mice against EAE induction.[32] Previous studies have demonstrated increased expression of the *IL17* gene in the spinal cord and elevated serum levels of this cytokine in EAE-affected mice.[5] Adoptive transfer of IL-17-producing cells induced EAE in healthy mice, and administration


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of antibodies against IL-17 interfered with the development and progression of EAE while delaying the onset of clinical symptoms.[33] In humans, IL-17 has been implicated in the demyelination process of nerve cells in patients with MS. Studies have shown a correlation between the severity of MS disease symptoms and an increase in the number of Th17 cells in the blood of patients. Elevated levels of IL-17 have also been observed in the CSF and brain lesions of MS patients.[34] In MS patients, the of the brain-blood barrier endothelial cells express the IL-17 receptor, and IL-17 binding to this receptor leads to a decrease in the expression of tight junction proteins and increased migration of $CD4^+$ T cells into the brain. Furthermore, IL-17 increases the expression of MMPs, resulting in blood-brain barrier dysfunction and neuron apoptosis.[35] Our study findings also support the increase of Th17 and IL-17 cells' importance in the development of EAE, consistent with previous studies. Therefore, suppressing the differentiation and proliferation of these cells could be a therapeutic goal for MS. Importantly, the transfer of Treg cells into EAE mice has been shown to prevent disease progression.[36] Treg cells play a pivotal role in maintaining immune homeostasis and tolerance. These cells employ mechanisms such as the production of inhibitory cytokines, inhibition of antigen-presenting cells' function, and induction of apoptosis to preserve immune tolerance. Notably, Tregs produce substantial amounts of inhibitory cytokines like TGF-β and IL-10, which effectively suppress the secretion of inflammatory cytokines from Th17 cells.[37] Dysregulation of Treg cell number and function has been observed in autoimmune diseases, including diabetes, polyglandular autoimmune syndrome type 2 (PAS-2), and MS.[38] Previous research has demonstrated that calcitriol can hinder the Th17 phenotype by inhibiting the transcription of *RORγt*, *IL17*, *IL23R*, and *IL22*,[39] while enhancing the Treg subset through the expression of *TGFB* and *FOXP3,* along with augmenting cytotoxic T-lymphocyte-associated protein 4 (CTLA-4).[40] In a study conducted in 2016 by Parastouei et al, the effects of calcitriol, either alone or combined with All-trans retinoic acid (ATRA), were examined in reducing EAE symptoms and altering gene expression in peripheral blood extracted from the spleen of mice. The dose and injection schedule used in our study were consistent with the calcitriol prevention Group alone (100 ng per mouse, every other day) in the mentioned study. Additionally, a study published in 2019 by Haghmorad et al, investigated the effect of different doses of calcitriol in the prevention and treatment of EAE. Their results indicated decreased expression of *RORγt* and *IL-17* genes and increased expression of *FOXP3*, *TGFB*, and *IL10* genes in spleen and lymph node lymphocytes of mice treated with medium (50 ng per mouse) and high (100 ng per mouse) doses of calcitriol. In the mentioned study, injections in prevention groups started two weeks before EAE induction and continued until day 25 after induction, while in treatment groups, injections were administered daily from the day after induction until the 25th day. Regarding the effect of calcitriol on Th17 cell differentiation and the expression of inflammatory cytokines, studies have revealed that calcitriol not only inhibits the activity but also the differentiation of Th17 cells. It has been shown that calcitriol suppresses Th17-related cytokines and transcription factors, including IL-17A, IL-17F, and RORC, in vitro when naive $CD4^+$ T cells differentiate into the Th17 lineage. Furthermore, when differentiated MOG-specific Th17 cells are transferred to mice in the presence of calcitriol, these cells exhibit reduced capability to induce EAE. This effect might be attributed, at least in part, to the decreased expression of CCR6, a chemokine receptor required for migration into the CNS.[40] Although the inhibitory effect of calcitriol on Th17 activity is well established, the underlying mechanisms are not fully understood. One study by Joshi and colleagues proposed that regulation of IL-17A may be mediated by direct binding of VDR to the IL-17A promoter. VDR-RXR complexes compete with nuclear factor of activated T cells (NFAT) for binding sites in the promoter, subsequently recruiting the transcription factor *RUNX1* and histone deacetylases (HDAC) to inhibit *IL17A* gene expression. This competition for the NFAT binding site also occurs in the promoter of the *IL2* gene, a primary target gene for calcitriol, indicating a potential general mechanism that applies to other genes regulated by NFAT. The involvement of HDACs suggests the involvement of epigenetic regulation in the inhibition of *IL17A* by calcitriol.[41] Regarding the effect of calcitriol on the induction of Treg cell differentiation, previous studies have demonstrated that calcitriol stimulates the production of $Foxp3^+$ Tregs in the spleen, lymph nodes, and spinal cord of mice with EAE.[42] The increase in *FOXP3* expression is directly related to its ability to inhibit the expression of *IL17* and *IFNG*, thereby exerting positive effects in the inhibition of EAE.

Moreover, the presence of TGF-β and its signaling plays a critical role in the inhibition of EAE by calcitriol.[43] Additionally, it has been shown that the inhibitory capacity of Tregs in MS patients is directly related to the serum level of 25 (OH) D3.[44] Calcitriol also enhances TGF-β induction in $CD4^+$ T cells cultured under neutral conditions or even in the presence of Th17 polarizing cytokines. In these cultures, calcitriol induces Foxp3 and CTLA-4, thereby augmenting the inhibitory capacity of Tregs.[42] Various mechanisms have been proposed through which calcitriol can induce a Treg-like phenotype, even in the context of Th17 polarization. Firstly, the VDR can bind to three vitamin D response elements (VDREs) in the conserved noncoding sequence of the *FOXP3* promoter, directly controlling the transcription of *FOXP3*.[45] Secondly, calcitriol reverses the inhibitory effect of Th17-polarizing cytokines on CTLA-4, leading to increased expression of CTLA-4. Finally, calcitriol upregulates the expression of indoleamine 2,3-dioxygenase (IDO), which also contributes to the increased number of Tregs.[46] This latter finding is interesting, as IDO has been reported to induce DCs, suggesting it may serve as a general target molecule of calcitriol in the immune system.

In general, in addition to the studies proposed by Parastouei et al, and Haghmorad et al, various other studies have demonstrated that administration of vitamin $D_3$ in the EAE model prevented the occurrence of clinical and pathological symptoms, significantly reduced Th1 and Th17 cells, while increasing the differentiation of Th2 and Treg cells.[47,48] These results highlight the ability of vitamin D to modulate inflammatory cell subsets towards a regulatory phenotype with inhibitory properties. Furthermore, in different studies, the levels of inflammatory cytokines IL-17, IFN-γ, TNF-α, IL-6, and IL-12 decreased in EAE mice receiving vitamin D, while the levels of inhibitory cytokines TGF-β and IL-10[49,50] were shown to increase in this animal model. Our study, consistent with other research, indicates that calcitriol can delay the onset and reduce the severity of clinical symptoms in EAE mice by altering the balance of Th17 and Treg towards Treg inhibitory cells.

In this research, we encountered specific limitations. One noteworthy limitation was the omission of serum vitamin D and calcium level measurements in the mice. Conversely, this project boasts several advantages, such as the application of an optimal vitamin D dosage and the utilization of a laboratory protocol previously established. Consequently, the need for trial-and-error experimentation in laboratory procedures was minimized. Furthermore, our study gained a significant advantage through the isolation of $CD4^+$ T cells from spleen cells with a purity exceeding 90%, thereby enhancing precision and setting it apart from prior investigations.

In this study, we investigated the impact of vitamin D (calcitriol) on the balance of Th17 and Treg cells and its role in regulating inflammation in the EAE mouse model. Additionally, we explored the influence of vitamin D on the expression of genes associated with Th9 cells, a relatively newly discovered cell type. Our results revealed that calcitriol effectively decreased the level of the inflammatory cytokine IL-17, while simultaneously increasing the level of inhibitory cytokine TGF-β in both healthy and EAE mice. Moreover, we made a novel observation by demonstrating that calcitriol can upregulate *IL9* gene expression in healthy and EAE mice, potentially contributing to the reduction of inflammation in the EAE mouse model. Further investigations are warranted to validate the anti-inflammatory role of IL-9 and explore potential therapeutic strategies aimed at enhancing this cytokine to mitigate inflammation in autoimmune diseases such as MS. In general, vitamin D exerts its anti-inflammatory effects in the animal model through diverse signaling pathways. Future studies should delve deeper into understanding these pathways.

## STATEMENT OF ETHICS

This study was approved by the Ethics Committee of Tehran University of Medical Sciences approved under the code number IR.TUMS.SPH.REC.1396.3158

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## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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