Iranian Journal of Basic Medical Sciences

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Vitamin D3 influence the Th1/Th2 ratio in C57BL/6 induced model of experimental autoimmune encephalomyelitis

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ARTICLEINFO

Article type:
Original article

Article history:
Received: Dec 26, 2013
Accepted: Apr 14, 2014

Keywords:
EAE
Th1/Th2 ratio
Vitamin D3

ABSTRACT

Objective(s): Multiple Sclerosis (MS) is known as a progressive inflammatory CNS disease. Cytokines belong to Th1 or Th2 family and inflammatory cells, play significant role in pathophysiology of MS. Thus, any treatment supposed to influence the relation between Th1 to Th2 cytokines expression. Although vitamin D has been prescribed as a therapeutic supplement of MS for a long time, it is not clear how much it may affect the Th1/Th2 ratio. To answer this question the present research was designed.

Materials and Methods: Thirty C57BL/6 adult female mice were used. The animals were randomly divided into trial and control groups. Experimental Autoimmune Encephalomyelitis (EAE) modeling for MS and clinical scoring as cited by others was used. Based on scoring and step of the disease vitamin D3 prescription (5 mg/kg) started and continued for three weeks.

Results: By using ELISA and RT-PCR the brain level of TNF-α, IL-10, IL-4 and IL-12 determined. Significant decrease of clinical symptoms in trial group which received vitamin D was seen comparing to control animals (P<0.05). The level of TNF-α but not IL-10 significantly decreased following vitamin D3 administration. By comparing the level of Th1 and Th2 Interleukins and counting the ratio of them we found that in treated animals the ratio was significantly less than non-treated (P =0.01).

Conclusion: According to the results, vitamin D3 may be able to suppress the inflammatory ways that lead to progression of MS. Whether this ability is clinically valuable in human subjects is not clear and needs more clinical research.

► *Please cite this paper as:*
Soleimani M, Jameie SB, Mehdizadeh M, Keradi M, Masumipoor M, Mehrabi S. Vitamin D3 influence the Th1/Th2 ratio in C57BL/6 induced model of experimental autoimmune encephalomyelitis. Iran J Basic Med Sci 2014; 17:785-792.

Introduction

Multiple sclerosis (MS) as chronic and neurodegenerative diseases is well known for its immune-mediated nature. Recent epidemiological reports confirm that the incidence of MS has been raised up dramatically. Because of the progressive and disabling nature of MS many researchers focused on its etiology, physiopathology, epidemiology and therapy (1). There are few hypotheses regarding the etiology and pathophysiology of MS. One of the up-to-date hypotheses mainly focuses on immune-inflammatory mediated nature of the disease. Accordingly, the beginning and progression of disease is due to the imbalance between the pro-inflammatory and anti-inflammatory cytokines that are produced by Th1 and Th2 cells (2). The history of it dates back to the late 80s when there were growing evidences of the importance of the role of various type of IL in MS (3). Despite the autoimmune base of the beginning of the disease, the continuation is mainly neurodegenerative. Regarding the role of T cells and their production, it is shown that the myelin proteins including myelin basic protein (MBP), myelin proteolipid protein (PLP) and myelin-oligodendrocyte glycoprotein (MOG) are attacked and destroyed by them. The presence of the pro-inflammatory cytokines including TNF-α and IL-12 in MS plaques are confirmed by immunhistochemical studies (4). Th1 cells secrete pro-inflammatory cytokines such as interferon-g (IFN-γ), tumor necrosis factor-a (TNF-α) and the Th2 release cytokines such as interleukin-4 (IL-4), IL-5 and IL-13 which respectively activate macrophages to clear intracellular pathogens and aid in antibody class-switching and removal of extracellular infectious agents. Among he certain type of interleukins, IL-10, IL-4, IL-12 and TNF-α are seem

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to be more important in onset, severity and progression of MS. IL-10 not only inhibits the production of other cytokines, such as IL-1 and TNF-a, but also ceases the proliferation of T cells (5). IL-4 inhibits the activation of Th1 cells and acts as a suppressor cytokine in Experimental Autoimmune Encephalomyelitis (EAE). (6) IL-12, critical for the differentiation of Th1 cells, was found to be elevated immediately prior to the onset of disease in EAE rat model (7). TNF-α production is associated with a Th1 response and classically induces activation of a variety of cell types and expression of adhesion molecules, chemokines, and cytokines (5). Kuroda *et al* in 1991 reported that injections of TNF-a lead to significant prolongation of clinical EAE and more severe cellular infiltration in the spinal cord (8). However, surprisingly, TNF-α-deficient mice develop a more severe form of EAE characterized by significantly more inflammation and demyelination (9). Beside to the hypothesis of Th1/Th2 imbalance, the role of mitochondrial dysfunction and ROS (reactive oxygen species) and free radicals production that leads to inflammatory response also considered in pathogenesis of MS. Consequently, using antioxidant such as vitamin D3 to suppress or decrease the rate and severity of the disease has received more attention in recent decades (10). The important role of vitamin D3 has been showed by clinical evidence. Low circulating levels of vitamin D3 have been found in MS patients especially during relapses stage of the disease. There are also some studies emphasize on the relation between geographical location and MS incidence rates which may be the result of a populations decreased exposure to UV radiation (11,12). Epidemiological studies show a lower MS incidence or mortality in temperate regions where vitamin D3 is abundant due to diets rich in fish oils, increased sun exposure, or high altitudes (13). Although the vitamin D3 hypothesis dates back to early 1970s, it got more important when it was shown that pharmacological doses of the functional metabolite of vitamin D3 can significantly reduce or eliminate the incidence of the disease in MS mouse EAE model (14). How vitamin D3 could be so effective is a question of two last decades, now we know that in addition to calcium homeostasis, vitamin D3 has strong immune modulating activity (15). It is shown that vitamin D3 mediates its function through a single vitamin D3 receptor (VDR) (16). In experimental studies daily administration of vitamin D3 before immunization prevents EAE from developing, while daily administration of the vitamin after the onset of the disease will only prevent disease progression (17). Although the role of VDR is well known, it seems that D3 also acts via other unknown mechanisms not solely through its receptors. Recently some mechanisms were suggested for the effectiveness of vitamin D3 in suppression or decrease the rate of progression of MS in animal model. In another study it is reported that adding vitamin D3 to the cultures of murine or human Peripheral blood mononuclear cell (PBMC), suppresses the release of typical Th1-type cytokines including IL-2, IFN-γ and TNF-α (18). Although the influence of vitamin D3 on expression and activity of certain types of interleukins has been reported by others, there is controversy regarding the relation between D3 administration and Th1/Th2 ratio in EAE model of MS. In order to answer how this ratio is affected by vitamin D3 administration the present research was designed.

Materials and Methods

Biological models

Thirty 10-12 weeks adult female C57BL/6 mice (18-20 g; Pasteur Institute of Iran) were used. The animals were randomly divided into four groups (n=6) including EAE, EAE + vitamin D3, EAE+ sesame oil and control. All the procedures used in this study were approved by The Committee of Ethics in Animal Research of Iran University of Medical Sciences.

Induction of EAE

To induce EAE, the routine procedures introduced by others were used as follows: The animals were immunized with 1:1 ratio of MOG 35-55 (Alexis, Switzerland) dissolved in Complete Freund's Adjuvant (CFA) containing 0.4 mg of mycobacterium tuberculosis (Sigma-Aldrich, USA). For this purpose 300 µg of MOG dissolved in 100µl PBS and mixed with equal volume of CFA. On day 0, each animal received 200 µl, two single shot, of MOG-CFA emulsion subcutaneously into two sites of the upper flanks. The supplement immune adjuvant, pertussis toxin (PTX), (Sigma-Aldrich, USA) at the dose of 200 µl containing 400 ng of PTX, was injected IP immediately after MOG-CFA emulsion, the same injection repeated 48 hr later.

Clinical EAE score

To approve the onset and stage of progression of the disease the standard scoring system sited elsewhere was used. Based on this method of scaling: clinical signs of no symptoms, distal weak or spastic tail, completely paralyased tail plus hind limb weakness, unilateral partial hind limb paralysis, bilateral partial hind limb paralysis, complete bilateral hind-limb paralysis, complete hind limb and unilateral partial forelimb paralysis, moribund and dead equaled to 0, 0.5, 1, 1.5, 2.0, 2.5, 3.0, 3.5, 4 and 5 respectively.

Vitamin D3 administration

Thereafter the animal showed score 1, intraperitoneal injection of vitamin D3 at the dose of 5 mg/kg in 150 µl sesame oil was started and continued for 3 weeks. For the control animals only sesame oil was used in the same volume and method.

Tissue preparation

Perfusion and fixation by aldehyde solutions was done transcardially via left ventricle. Brains were removed and post-fixed in the same solution overnight. After that the brains were exposed to tissue processing and in turn paraffin embedding. By using rotatory microtome coronal sections of 4 µ were prepared. To study the myelination in certain part of the brain including corpus callosum, Luxol Fast Blue staining with Nissl contra staining was used. The total surface of demyelinated regions was calculated by version 4.6 Infinity Software. Based on allen mouse brain atlas, in a rostrocaudal pattern a total number of 20 cross sections of 4 µ of corpus callosum with interval of 150 µ per mouse were observed and studied. To calculate the ratio of demyelinated surface to myelinated area first the total area of corpus callosum was calculated by using special effects of software, with the same procedure the demyelinated area was also computed then the difference was presented in percentage. The total number of 20 cross-sections of corpus callosum was prepared and the mean of them were calculated.

Study of the level of cytokine

The animals were sacrificed by lethal dose of ketamine and xylazine. Brain tissue was removed and rapidly transferred to liquid nitrogen. To homogenize the tissue, a solution including (1x Lysis Buffer: 1x TBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide.) combine with 10 µl PMSF solution, 10 µl sodium orthovanadate solution and 10–20 µl protease inhibitor cocktail solution per ml of 1x RIPA lysis buffer were mixed to prepare complete RIPA (Santa Cruze). Three ml of complete RIPA per gram of tissue brain homogenate was spun at 10,000× g for 10 min at 4˚C and supernatants were collected and stored at −70˚C. The concentration of IL-12, TNF-α, IL-4 and IL-10 in the supernatants of brain extraction, at 1:10 dilution in 1% BSA in phosphate buffered saline (PBS), was assayed in an ELISA set-up using commercially available antibodies according to the procedures supplied by the manufacturer (eBiosciences, Austral).

RNA extraction and RT-PCR

Animals were sacrificed by decapitation within a few seconds after being picked up from their home cage. Brain was removed using aseptic techniques, placed in sterile tubes and frozen on dry ice. Total RNA extraction was performed using RNX-plus (Cinnagen, Iran) according to the protocol. The RNA samples were re-suspended in 30 µl of nuclease-free water. The concentration and quantification of total RNA was measured with spectrophotometer, with the OD260/OD280 ratio of all RNA samples 1.9–2.0 and OD260/OD230 ratio up to 2. The first strand cDNA was synthesized with the First Strand cDNA Synthesis Kit (Bioneer kit, K-2101, Korea). For each reaction, 1 µg (1 µl) RNA was used for reverse transcription, in a mixture of 20 pmoles (1 µl) random primer, and 18 µl Diethylpyrocarbonate-Water (DEPC-W) with a final volume of 20 µl. The mixture was incubated at 15˚C for 1 min, 50˚C for 60 min, and heated at 95˚C for 5 min to terminate the reaction. The cDNA was subsequently stored at −20˚C. qPCR was performed with 1 µl of primer (10 pmole), 1 µl of template, 3 µl of DEPC.D.W and 5 µl mastermix (AccuPower® 2X GreenStarTMqPCRmaster mix, Bioneer kit, Korea). All PCR reactions were performed in the following condition: initial 95˚C for 15 min followed by 40 cycles at 95˚C for 15 sec and 60˚C for 30 sec. The PCR primers for each gene were shown in Table 1. Each sample was tested in duplicated. The values were normalized against the housekeeping genes GAPDH (glyceraldehyde-3phosphatedehydrogenase). The CT-value is an important quantitative parameter in real-time PCR analysis. All RT-PCR reactions were carried out in triplicate and with no template control. The ΔCT of the controls was used as the calibrator. The fold change was calculated according to the formula $2^{-(\Delta\Delta CT)}$, where ΔΔCT is the difference between ΔCT and the ΔCT calibrator value.

Statistical analysis

By using SPSS 21 and statistical exams, data analyzed and presented as mean ± SD.

The result of the real time PCR was analyzed by two sided Student's t-test. *P-value* less than 0.05 were considered significant.

Results

Scoring

Loss of weight which was considered as one of the important markers for confirmation of model, significantly occurred in EAE induced animals comparing to control and sham vehicle. The maximum mean score for the EAE + vitamin D3 animals was significantly lower than the animals of EAE ($P< 0.05$, 2.2±0.9, 1.4±0.41 respectively).

Histological study

EAE caused significant demyelination in certain

Table 1. Nucleotides sequence of the forward and reverse primers for the RT-PCR

Cytokines	Forward	Reverse
Necrosis Factor (TNF-α)	5'- GCCCACGTCGTAGCAAACC -3'	5'- GTCTTTGAGATCCATGCCGTTG -3'
Interleukin-10 (IL-10)	5'- GCGCTGTCATCGATTTCTCC -3'	5'- TGGCCTTGTAGACACCTTGG -3'
Interleukin-4 (IL-4)	5'- GTCACAGGAGAAGGGACGC -3'	5'- AAGCACCTTGGAAGCCCTAC -3'
Interleukin-12(IL-12) subunit beta	5'- TGTCGCTAACTCCCTGCATC -3'	5'- CTGAGGACACATCCCACTCC -3'
Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)	5'- TTGTGCAGTGCCAGCCTC -3'	5'-CAACAATCTCCACTTTGCCACT -3'

Table 2. Expression of mRNA analyzed by REST software

Group	IL-4			IL-10			IL-12			TNF-α		
	Exp	P(H1)	Result	Exp	P(H1)	Result	Exp	P(H1)	Result	Exp	P(H1)	Result
Brain Control- EAE	0.831	0.682		0.589	0.045	DOWN	2.387	0.023	UP	6.052	0.004	UP
Brain Control - EAE+Sesame oil	2.136	0.056		1.803	0.01	UP	2.334	0	UP	1.31	0.317	
Brain Control - EAE+vitamin D3	1.225	0.579		0.951	0.851		1.625	0.172		2.103	0.008	UP
Brain EAE - EAE+ vitamin D3	1.474	0.269		1.613	0.176		0.681	0.117		0.347	0.004	DOWN
Brain EAE- EAE+ Sesame oil	2.571	0.041	UP	3.058	0.01	UP	0.978	0.914		0.217	0	DOWN
Brain Oil - EAE+ Sesame oil	1.063	0.877		0.782	0.59		2.301	0.018	UP	1.556	0.189	
Brain D3 - EAE+ vitamin D3	0.706	0.05	DOWN	0.459	0.261		2.028	0.111		4.077	0.007	UP

Exp is related to mRNA expression, P(H1) shows Pv, and Result is correlated with decrease or increase in gene expression. UP: up-regulation, DOWN: down-regulation

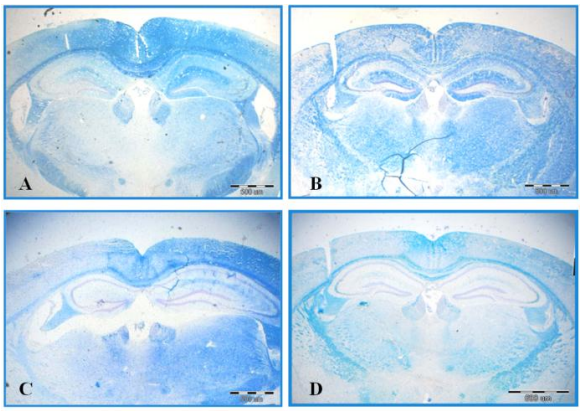

Figure 1. Luxol Fast Blue staining that was used for rate of demyelination showed significant differences among groups including control (A), EAE (B), EAE + sesame oil and EAE + Vitamin D3. Demyelination area showed by dots. Control is 3.08±0.67

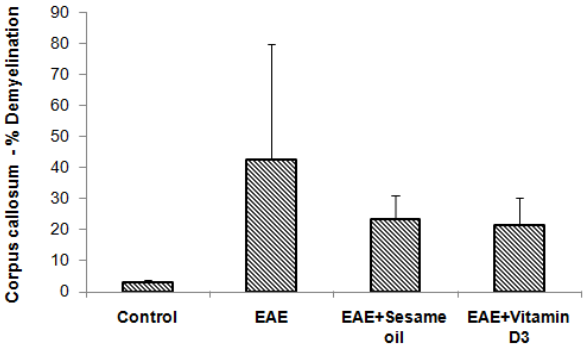

Figure 2. Histogram of demyelination in corpus callosum showed significant difference between EAE and EAE+ sesame oil and EAE + Vitamin D3. The difference between EAE + vitamin D3 and EAE + sesame oil is not significant

area of the brain like corpus callosum. Comparing EAE group (42.56±37.12 %) with the animals of EAE+ vitamin D3 (21.40±8.92%) showed significant less demyelination in later group (Figure 1). For the animals received only sesame oil the result showed significantly less demyelination comparing with EAE and EAE + vitamin D3 (Figure 2).

Cytokine analysis

Level of brain cytokines including IL-12, TNF-α (Th1)and IL-10, IL-4 (Th2) was measured on day 21 post-immunization. Level of TNF-α and IL-12 was higher but not significant in untreated group

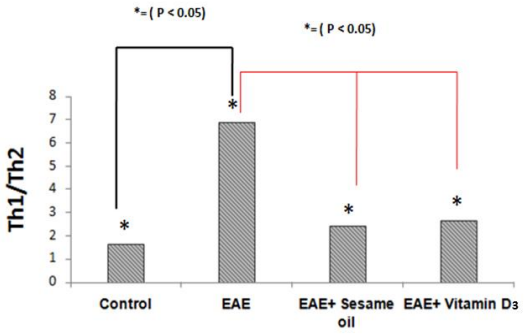

Figure 3. Histogram of the Th1/Th2 ratio, higher ratio was seen in EAE comparing with other groups

compared with the control animals (Figure 3). Our finding showed that vitamin D3 administration significantly decreased level of TNF-α comparing with untreated animal (P<0.01) (Figure 3), this finding is the same for control animals (P<0.05) (Figure 3). Conversely, elevated level of IL-10 was found in treated animals comparing with not treated (P<0.001) and control ones (P<0.05) (Figure 4). Regarding the level of IL-12 and IL-4, no significant difference was observed between untreated and control groups. Put these findings together showed that the ratio of Th1/Th2 in EAE animals was higher than sesame and vitamin D3 treated animals. Comparing to control the decrease ratio of Th1/Th2 might be the result of more expression of Th2 cytokines or less expression of Th1.

Cytokine gene expression

Pro-inflammatory cytokines expression analyzed with REST software (Table 2). IL-12 mRNA and TNF-α mRNA expression were increased remarkably in EAE model. Comparing control group, significant increase of IL-4 anti-inflammatory cytokines mRNA expression in EAE was found. Strong increasing expression of IL-10 mRNA did not occurred in EAE animals. However, a noticeable but not significant decline of IL-10 mRNA expression was seen in sesame and vitamin D3 treatment group. Compared to control group, IL-4 mRNA expression in sesame oil group was obviously higher. Regarding IL-4 mRNA expression, it was obviously expressed lower in EAE mice. Significant lower expression of TNF-α mRNA was also observed in the vitamin D3 and sesame oil (Figure 5).

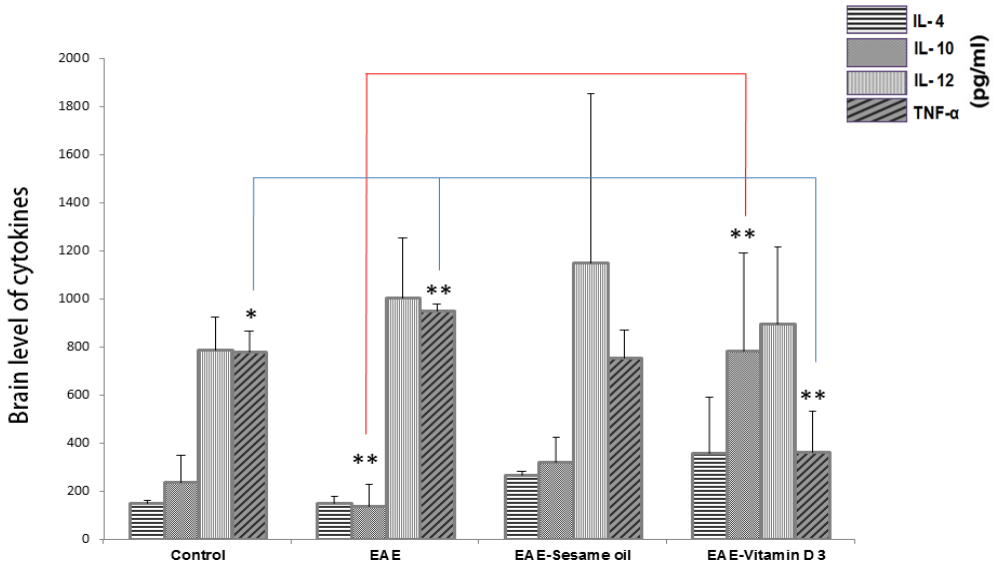

Figure 4. Level of cytokines in trial and experimental groups, IL-10 in EAE significantly decreased comparing with control, for two other groups (EAE-sesame oil) and (EAE-vitamin D3) this level increased. Level of TNF-α showed decreased after using therapy

Discussion

Autoimmunity has major role in MS and most therapeutic strategies emphasize on using immunomodulatory and immunosuppressive agents. The role of interleukins studied in two aspects, some researchers mainly emphasized on certain interleukins such as IL-6, IL-10, IL-4, etc. and others mainly focused on the ratio of the interleukins produced by Th1 and Th2. We studied both aspects and showed that the ratio of Th1 to Th2 has been changed dramatically in animals with MS and also in vitamin D treated animals. The elevated serum level of Th1 cytokines such as IL-12 was reported by Kaplan *et al* Elevation of IFN-γ (Th1) and IL-4 (Th2) also showed in progressive MS (19, 20). Issazadeh *et al* (7) reported that the expression of IL-4 significantly decreased in EAE that is the same of what we found. IL-4 mRNA was undetectable until disease reduction in Swiss/Jackson Laboratory mice (SJL mice) immunized with PLP (21). Some recent studies showed that IL-4 has been implicated as a suppressor cytokine in EAE. Controversy about IL-4 still remains unsolved and our data showed unchanged level of IL-4 in EAE, although it increased not-significantly after treatment by vitamin D. However, little expression of IL-4 has been reported in other models of EAE according to Issazadeh *et al* (7). Regarding IL-10 that inhibits the production of IL-1 and TNF-α, there are enough evidences that IL-10 decreased in MS patients and EAE model (7). Cannella *et al* (22) showed that the administration of anti-IL-10 monoclonal antibody in murine EAE model increased the severity of disease. Comparing the role of IL-10 with IL-4 there are some reports that showed the severity of disease in C57BL/6 IL-10 deficient is more than in IL-4 deficient mice, Bettelli *et al* (6). The pro-inflammatory role of TNF-α and IL-12 is well known. Overexpression of TNF-α in transgenic mouse, lead to oligodendrocyte apoptosis and demyelination, the absence of that, might improve EAE, Akassoglou *et al* (23). Elevated serum of TNF-α have been reported in MS patients. Similar to what was reported by Zhou *et al* (24), the expression of IL-10 elevated during the course of MS; we also found the same result. Based on the literature, IL-10 is capable of inhibiting the synthesis of pro-inflammatory cytokines like IFN-γ, IL-2, IL-3, and TNFα made by cells such as macrophages and regulatory T-cells and thus promotes survival of neurons and all glial cells in the brain by blocking the effects of pro-apoptotic cytokines and by promoting expression of cell survival signals (25). IL-10 also inhibits inflammation via three major pathways including reducing synthesis of proinflammatory cytokines, suppressing cytokine receptor expression, and inhibiting receptor activation, Zhou *et al* (26). By attention to the hypothesis of the imbalance between the family cytokines produced by Th1 and Th2, it is rational that any agents that could restore the ratio balance of Th1/Th2 might be a suitable choice for therapy in MS, Eikelenboom *et al* (2). In this regard, it is shown that certain drugs for MS such as IFN-β act via rebalancing of TNF-α and IL-10 (24).

During the last decade the role of vitamin D3 as therapy or prophylaxis of MS revived more attention, epidemiological study showed the correlation between prevalence and the incidence of MS with the geographical location and diet. Low exposure to sun light and diet with low vitamin D3 leads to increase the rate and severity of MS. Now we know that D3 not only easily passes the blood-brain barrier but

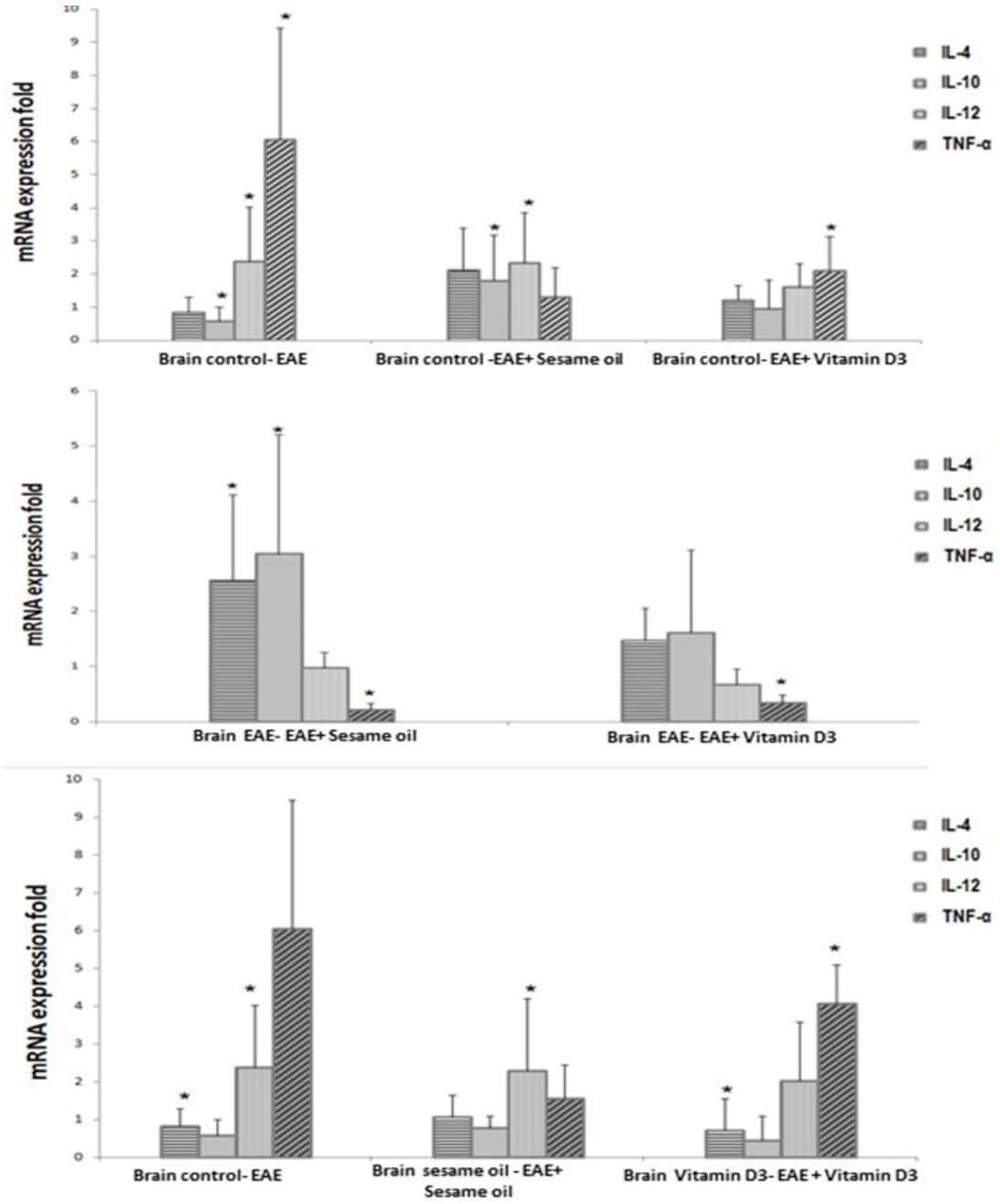

Figure 5.mRNA expression fold change in inflammatory genes of EAE mice with REST software increase vs. anti-inflammatory genes. The result reversed after treatment

also is produced by nervous system constituent cells, Garcion *et al* (26). Vitamin D3 exerts molecular and behavioral effects on various neuronal and non-neuronal cells that expressing its receptor, VDR. These observations confirmed that during ongoing EAE, D3 might display both immunomodulatory and neurological effects and limiting the disease (26). How vitamin D3 shows such ability is not known well. However, there are some hypotheses that show the mechanisms that vtamin D may act. Vitamin D has general immunomodulatory and anti-inflammatory effects not only by reducing DCs, Th1, Th17, B-cell proliferation and proinflammatory cytokines but also by promoting Th2 phenotype, anti-inflammatory cytokines. Vitamin D also re-equilibrates the balance between Th1 and Th2 cells, resulting in a reduction of inflammation, Cantorna *et al* (27) and Smolders *et al* (15). The discovery of VDR in the rat forebrain, hippocampus, cerebellum, brainstem, spinal cord, and perivascular tissue and the discovery of 1a-OHase in cerebellum and cerebral cortex, Van Amerongen *et al* and Zehnder *et al* opened many ways to explain how vitamin D3 could target the neurons (28, 29). Profound alterations in the brain at birth have been demonstrated in rats born from vitamin D3-deficient

mothers (30). Although, Garicon *et al* reviewed the influences of D3 on neurons, oligodendrocyte, as well as astrocytes, the exact pathways of these effects remains to be established (31). According to their results vitamin D3 seems to be neuroprotective and anti-inflammatory. In vitamin D treated EAE rats, reduced number of infiltrated macrophages in nervous tissue was reported. It seems that vitamin D3 could suppress the trans-endothelial migration of monocytes, Nashold *et al* (32). They also showed that vitamin D3 could inhibit inflammatory response in MS via activating Rag-1 not by inhibiting Th1 response.

Muthian *et al* (33) suggested the same role for vitamin D3 to modulate Th1 response throughout inhibiting JAK-STAT signaling transfer pathway. Despite some known or postulated mechanisms of vitamin D3 on EAE model of MS or MS it seems that the behavior of D3 regarding different cytokines of Th1 or Th2 are different. For example, based on our results, IL-4 increased not significantly in EAE animals following vitamin D administration, although it is not near to the desirable therapeutic level for IL-4 as an anti-inflammatory, it showed the possible optimum effects of vitamin D3. Comparing our results with studies of Nashold *et al* (34) that showed the increasing level of IL-4 in EAE animals treated by vitamin D3, we assume the tissue of study, dose of vitamin D3 and the method of administration could be the matter of difference. Also similar to our result, Mosayebi *et al* (35) reported IL-10 increased after injection vitamin D3 in EAE model. We also found that the level of the TNF-α significantly decreased following vitamin D3 administration. It is possible that vitamin D3 playsan important role to enhance the effects of Anti-inflammatory cytokines.

Regarding our results for sesame oil as vehicle we found the effectiveness which agreed with the results reported by Ghazavi *et al* (36). The exact mechanisms of the vehicle are not established yet, although they believed on the role of sesame oil on reducing activity of Th1 or inducing Th2. We are not sure on this mechanism, so we suggest using other vehicle to exclude the effects of sesame oil. Further study needs to focus on the mechanism of sesame oil effectiveness.

With attention to immunoinflammatory basis of the beginning of the disease, the above mechanisms, if not completely, to some extent could explain the onset of MS. However, the continuation and progression of disease is mainly neurodegenerative i.e. demyelination of certain area of central nervous system. The histological findings of our research confirmed the same findings reported by the others. In fact the immunoinflammatory process leads to the destruction of myelin (37). It is not clear whether the variety of the structure of myelin in CNS including its proteins could affect this process or not and needs more research. Putting these together it is logical that any intervention could be able to cease or decrease the rate of first step of disease i.e. immunoinflammatory part, also could stop the later step or neurodegenerative part.

Conclusion

Based on our findings vitamin D3 should consider in prophylaxis for the high- risk population.

Acknowledgment

The present research work was done in Basic Science Lab, Department of Medical Basic Sciences Faculty of Allied Medicine of Iran University of Medical Sciences, Tehran, Iran.

References

1. Sloka S, Silva C, Wang J, Yong V W. Predominance of Th2 polarization by vitamin D through a STAT6-dependent mechanism. J Neuroinflammation 2011; 8:56.
2. Eikelenboom MJ, Killestein J, Kragt JJ, Uitdehaag BM, Polman CH. Gender differences in multiple sclerosis: cytokines and vitamin D. J Neurol Sci 2009; 286:40-42.
3. Kidd P. Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. Altern Med Rev 2003; 8:223-246.
4. Sospedra M, Martin R. Immunology of multiple sclerosis. Annu Rev Immunol 2005; 23:683-747.
5. Begolka WS, Vanderlugt CL, Rahbe SM, Miller SD. Differential expression of inflammatory cytokines parallels progression of central nervous system pathology in two clinically distinct models of multiple sclerosis. J Immunol 1998; 161:4437-4446.
6. Bettelli E, Das MP, Howard ED, Weiner HL, Sobel RA, Kuchroo VK. IL-10 is critical in the regulation of autoimmune encephalomyelitis as demonstrated by studies of IL-10- and IL-4- deficient and transgenic mice. J Immunol 1998; 161:3299-3306.
7. Issazadeh S, Ljungdahl A, Höjeberg B, Mustafa M, Olsson T. Cytokine production in the central nervous system of Lewis rats with experimental autoimmune encephalomyelitis: dynamics of mRNA expression for interleukin-10, interleukin-12, cytolysin, tumor necrosis factor alpha and tumor necrosis factor beta. J Neuroimmunol 1995; 61:205-212.
8. Kuroda Y, Shimamoto Y.Human tumor necrosis factor-alpha augments experimental allergic encephalomyelitis in rats. J Neuroimmunol 1991; 34:159-164.
9. Liu J, Marino MW, Wong G, Grail D, Dunn A, Bettadapura J, *et al*. TNF is a potent anti-inflammatory cytokine in autoimmunemediated demyelination. Nat Med 1998; 4:78-83.
10. Wiseman H. Vitamin D is a membrane antioxidant. Ability to inhibit iron-dependent lipid peroxidation in liposomes compared to cholesterol, ergosterol and tamoxifen and relevance to anticancer action. FEBS Lett 1993; 327:285-288.
11. Freedman DM, Dosemeci M, Alavanja MC. Mortality from multiple sclerosis and exposure to residential and occupational solar radiation: a case-control study based on death certificates. Occup Environ Med 2000; 57:418-421.

12. Van der Mei IA, Ponsonby AL, Blizzard L, Dwyer T. Regional variation in multiple sclerosis prevalence in Australia and its association with ambient ultraviolet radiation. Neuroepidemiology 2001; 20:168-174.
13. Esparza ML, Sasaki S, Kesteloot H. Nutrition, latitude, and multiple sclerosis mortality: an ecologic study. Am J Epidemiol 1995; 142:733-737.
14. Goldberg P. Multiple sclerosis: vitamin D and calcium as environmental determinants of prevalence. Sunlight, dietary factors and epidemiology. Int J Environ Stud 1974; 6:19-27.
15. Smolders J, Damoiseaux J, Menheere P, Hupperts R. Vitamin D as an immune modulator in multiple sclerosis, a review. J Neuroimmunol 2008; 194:7-17.
16. Sioka C, Kyritsis AP, Fotopoulos A. Multiple sclerosis, osteoporosis, and vitamin D. J NeurolSci 2009; 287:1-6.
17. Cantorna MT, Hayes CE, DeLuca HF. 1,25-Dihydroxyvitamin D3 reversibly blocks the progression of relapsing encephalomyelitis, a model of multiple sclerosis. Proc Natl Acad Sci USA 1996; 93:7861-7864.
18. Rausch-Fan X, Leutmezer F, Willheim M, Spittler A, Bohle B, Ebner C, *et al*. Regulation of cytokine production in human peripheral blood mononuclear cells and allergen-specific th cell clones by 1alpha,25-dihydroxyvitamin D3. Int Arch Allergy Immunol 2002; 128:33-41.
19. Lafaille JJ. The role of helper T cell subsets in autoimmune diseases. Cytokine Growth Factor Rev 1998; 9:139-151.
20. Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunology Today 1996; 17:138-146.
21. Begolka WS, Miller SD. Cytokines as intrinsic and exogenous regulators of pathogenesis in experimental autoimmune encephalomyelitis. Res Immunol 1998; 149:771-781.
22. Brosnan CF, Cannella B, Battistini L, Raine CS. Cytokine localization in multiple sclerosis lesions: correlation with adhesion molecule expression and reactive nitrogen species. Neurology 1995; 45:S16-S21.
23. Akassoglou K, Bauer J, Kassiotis G, Pasparakis M, Lassmann H, Kollias G, *et al.* Oligodendrocyte apoptosis and primary demyelination induced by local TNF/p55TNF receptor signaling in the central nervous system of transgenic mice: models for multiple sclerosis with primary oligodendrogliopathy. Am J Pathol 1998; 153:801-813.
24. Strle K, Zhou JH, Shen WH, Broussard SR, Johnson RW, Freund GG, *et al*. Interleukin -10 in the brain. Crit Rev Immunol 2001; 21:427-449.
25. Cammer W. Protection of cultured oligodendrocytes against tumor necrosis factor-alpha by the antioxidants coenzyme Q (10) and N-acetyl cysteine. Brain Res Bull. 2002; 58:587-592.
26. Garcion E, Sindji L, Nataf S, Brachet P, Darcy F, Montero-Menei CN. Treatment of experimental autoimmune encephalomyelitis in rat by 1, 25-dihydroxyvitamin D3 leads to early effects within the central nervous system. Acta Neuropathol 2003; 105:438-448.
27. Cantorna M. Vitamin D and its role in immunology: multiple sclerosis and inflammatory bowel disease. ProgBiophysMolBiol 2006; 92:60-64.
28. Van Amerongen BM, Dijkstra CD, Lips P, Polman CH. Multiple sclerosis and vitamin D: an update. Eur J Clin Nutr 2004; 58:1095-1109.
29. Zehnder D, Bland R, Williams MC, McNinch RW, Howie AJ, Stewart PM, *et al*. Extrarenal expression of 25-hydroxy vitamin D(3)-1 alpha-hydroxylase. J Clin Endocrinol Metab 2001; 86:888–894.
30. Eyles D, Brown J, Mackay-Sim A, McGrath J,Feron F. Vitamin D3 and brain development. Neuroscience 2003; 118:641-653.
31. Garcion E, Nataf S, Berod A, Darcy F, Brachet P. 1,25- Dihydroxyvitamin D3 inhibits the expression of inducible nitric oxide synthase in rat central nervous system during experimental allergic encephalomyelitis. Mol Brain Res 1997; 45:255-267.
32. Nashold FE, Hoag KA, Goverman J, Hayes CE. Rag-1-dependent cells are necessary for 1,25-dihydroxyvitamin D(3) prevention of experimental autoimmune encephalomyelitis. J Neuroimmunol 2001; 119:16-29.
33. Muthian G, Raikwar HP, Rajasingh J, Bright JJ. 1,25 Dihydroxyvitamin-D3 modulates JAK-STAT pathway in IL-12/IFNgama axis leading to Th1 response in experimental allergic encephalomyelitis. J Neurosci Res 2006; 83:1299-1309.
34. Nashold F, Miller D, Hayes C.1,25-Dihydroxyvitamin D treatment decreases macrophage accumulation in the CNS of mice with experimental autoimmune encephalomyelitis. J Neuroimmunol 2000; 103:171-179.
35. Mosayebi G, Ghazavi A, Payani MA. The effect of vitamin D3 on the inhibition of experimental autoimmune encephalomyelitis in C57BL/6 mice. RJMS 2006; 13:189-196
36. Ghazavi A, Mosayebi G. The mechanism of sesame oil in ameliorating experimental autoimmune encephalomyelitis in C57BL/6 mice. Phytother Res 2012; 26: 34-38.
37. Wergeland S, Torkildsen O, Myhr K-M, Aksnes L, Mork SJ. Dietary vitamin D3 supplements reduce demyelination in the cuprizone model. PLoS One 2011; 6:e26262.