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Therapeutic Effect of Vitamin D3 in Multiple Sclerosis Patients

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Multiple sclerosis (MS) is an inflammatory disease in which the myelin sheaths around the axons of the central nervous system are damaged. The damage leads to demyelination and scarring as well as a broad spectrum of signs and symptoms. The epidemiological data suggest a possible influence of vitamin D as an immunomodulatory agent on multiple sclerosis susceptibility as well as on clinical course of the disease. We investigated the effects of short-term vitamin D3 therapy on Iranian patients with MS. In a prospective randomized controlled trial study, 62 MS patients received 300,000 IU/month vitamin D3 or placebo as intramuscular injection for 6 months. Our results showed no significant difference between the treatment and the control groups in the expanded disability status scale scores and number of gadolinium-enhancing lesions during the 6-month treatment period. After 6 months, the levels of cell proliferation in the vitamin D treatment group were significantly lower than the control group. Also, the levels of transforming growth factor-beta and interleukin-10 in the vitamin D treatment group were significantly higher than the control group. This result suggests that vitamin D therapy may help prevent the development of MS and could be a useful addition to the therapy.

Keywords Multiple Sclerosis, Vitamin D3, Cytokines, Cell Proliferation.

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) (Dhib-Jalbut, 2007; Korn, 2008; Raine et al.,

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1996). The etiology and pathogenesis of MS are uncertain, but genetic and environmental factors are involved in MS (Brosnan and Raine, 1996; Winquist et al., 2007; Zuvich et al., 2009). Several studies have shown that one potential non-genetic factor affecting the development of MS is vitamin D status (Pierrot-Deseilligny, 2009; Smolders et al., 2008a) The evidence comes firstly from ecological studies that show an inverse association between ambient levels of ultraviolet B (UV-B) radiation, which is essential for vitamin D synthesis, and MS prevalence or mortality (van der Mei et al., 2001). In addition, lower vitamin D intake or serum 25-hydroxyvitamin D (25(OH)D) concentrations were associated with increased MS risk (Munger et al., 2004, 2006).

For instance, low blood levels of 25(OH)D have been reported in 50 to 70% of patients in different MS populations (Myhr, 2009; Nieves et al., 1994; Ozgocmen et al., 2005). Another example establishing this association was provided by Soilu-Hanninen et al. (2005) who have demonstrated that summer serum levels of 25(OH)D were significantly lower in newly diagnosed Finnish MS patients than in controls. Also, the severity of MS has been shown to associate with 25(OH)D levels (Smolders et al., 2008b). However, lower 25(OH)D levels have also been reported during relapses in relapsing remitting MS patients (Soilu-Hanninen et al., 2005).

Yet, the exact mechanism how vitamin D may be linked to MS is not yet clear. Vitamin D displays anti-inflammatory and immunomodulatory properties (Smolders et al., 2008a). Most of the biological effects of the activated form of vitamin D (calcitriol; 1,25-dihydroxycholecalciferol or $1,25(OH)_2D$) are mediated by vitamin D receptor. This receptor is expressed by most cell types of the immune system such as monocytes, antigen-presenting cells, and activated lymphocytes (Provvedini et al., 1983; Veldman et al., 2000). Activation of the vitamin D receptor modulates alteration of TH1 and TH2 cytokines production and lymphocyte proliferation. Vitamin D inhibits B and T lymphocyte proliferation and reduces the production of inflammatory-, and MS-associated cytokines.

Several studies on experimental autoimmune encephalomyelitis (EAE), which is an animal model of MS, have illustrated that supplementation with active vitamin D blocks the onset and progression of EAE (Cantorna et al., 1996; Lemire and Archer, 1991; Muthian et al., 2006). There are also some indications of disease-modifying effects of vitamin D in MS. A safety study on 13 MS patients showed that oral calcitriol (2.5 μg/day) is safe and well tolerated for one year by diet-compliant relapsing-remitting MS patients (Wingerchuk et al., 2005).

Supplementation of 25 μg vitamin D (in 39 MS patients) for 6 months led to an increase of plasma 25(OH)D, a significant increase of the cytokine TGF-β and a decline in IL-2 mRNA levels (Mahon et al., 2003) A small study in 12 patients with supplementation of up to 1000 μg/day vitamin D for 28 weeks showed a decline in the number of gadolinium enhancing lesion on MRI per

patient (Kimball et al., 2007). Although there is evidence to support the role of vitamin D as an immune-suppressing agent and the possibility that it may influence MS susceptibility, yet more double–blind placebo-controlled studies are necessary to determine the potential of vitamin D as an immune modulator in MS.

MATERIALS AND METHODS

Patients

Patients were diagnosed with MS by the McDonald criteria (McDonald et al., 2001). The patients were recruited from October 2009 to April 2010. Inclusion criteria were Iranian patients with MS and: (i) at least 1 relapse in the previous 12 months; more than 3 lesions on spinal or brain-MRI or both, (ii) baseline expanded disability status scale (EDSS) from 0 to 3.5, and (iii) age from 18–60 years. Exclusion criteria were: clinically isolated syndrome (CIS), progressive MS; MS patients with clinical relapses occurring during the study, drug abuse, use of digitalis or vitamin D supplementation; any condition predisposing to hypercalcaemia; nephrolithiasis or renal insufficiency; pregnancy or unwillingness to use contraception; and unwillingness to restrict dietary calcium (Wingerchuk et al., 2005). The study was performed in keeping with the Helsinki declaration on research with human subjects, and the protocol approved by the institutional ethical committee (AUMSEC-85-13/7).

Treatment

Patients aged 18 to 60 years from hospitals of Medical University of Arak, were randomized independently in a double-blind design into one of two treatment groups. Vitamin D3-treated individuals (n = 28) received 300,000 IU vitamin D3 every month as intra-muscular injection and control group (n = 34) received placebo as intra-muscular injection. All treatments were repeated for 6 months and all patients received Interferon B-1a (Cinnovex, Fraunhofer Institute, Germany) as standard treatment. All clinical and immunological variables and level of 25(OH)D were measured at the baseline and 1 month after last injection.

Vitamin D Assessment

Blood withdrawal was performed in the period from October 2009 to March 2010. The serum samples were collected before and after treatment (1 month after last injection). The collected serum was immediately shielded from directed light and stored at −20°C. All samples were analyzed simultaneously for 25-hydroxyvitamin D (25(OH)D) by enzyme immunoassay using

kits according to the manufacturer's instructions (Immunodiagnostic Systems, Inc.). Briefly, calibrator or samples added to the appropriate wells of the antibody coated plate in duplicate. The plate incubated at room temperature for 2 hours. Then, the plate washed and enzyme conjugate added to all wells. The plate incubated at room temperature for 30 minutes. Tetramethylbenzidine (TMB) substrate added to all wells. The absorbance of samples measured at 450nm (reference 650nm) using a microplate reader (Stat Fax 2100, USA). 25(OH)D calibrators are standardized using U.V. quantification. The sensitivity, defined as the concentration corresponding to the mean minus 2 standard deviations of 10 replicates of zero calibrator, was 5 nmol/L.

Neurologic Evaluation

Patients were evaluated every 2 months by means of EDSS. The MRI brain scans with contrast enhancement were performed in the beginning of the study and at the end. Throughout the study, the neurologist, physician and the radiologist were unaware of the treatment assigned to each patient.

Immunologic Evaluation

Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Isopaque. The blood samples were diluted 1:1 in RPMI 1640 contains 5 mM HEPES, 100 U/mL of penicillin and 100 μg/mL of streptomycin (all obtained from Gibco, Life Technologies, Inc., Gaithersburg, Md.). PBMC were isolated by centrifugation (600*g* for 20 min) with 1.077 g/mL Ficoll-Isopaque (Lymphoprep, Nyegaard, Oslo, Norway) and washed in RPMI with 10% heat-inactivated fetal bovine serum (FBS). The number of viable cells was counted by trypan blue exclusion. The cells were then resuspended in RPMI supplemented with 10% FBS and used for proliferation assay and cytokine determination as described below.

Proliferation of PBMC

Proliferation was checked by the MTT assay method. A total of 3×10^3 cells in 200μl RPMI 1640 supplemented with 10% FBS were stimulated with 1μg/mL PHA. The plates were then incubated in a 5% CO_2 at 37 °C for 72 h. Twenty microliters of 5 mg/mL MTT (3-(4,5-dimethyldiazol-2-yl)-2,5-dipenyl; Sigma-Aldrich, St. Louis, MO) were added to the cells, followed by incubation for 4h. After centrifugation, the medium was removed, and 200μl of DMSO were added to each well. The optical density (OD) values of stimulated and non-stimulated cells were measured at 540 nm using a microtiter plate reader (Stat Fax2100, USA). All experiments were performed in triplicates. Proliferation responses for the MTT assay were expressed in terms of the mean

stimulation index (SI) and obtained by dividing the OD values of stimulated cells by the respective OD values of the non-stimulated ones.

Cytokines Assay

The PBMC at a density of 2×10^6 cells/mL were incubated in 1-mL cultures once in presence, and once in absence, of PHA (1 μg/mL) and cultured in cell culture condition for 72 h. The supernatants were collected and interferon gamma (IFN-γ), interleukin-10 (IL-10) and transforming growth factor -beta (TGF-β) were quantified by ELISA kit (R&D Systems) according to the manufacturer's protocol. Each sample was tested in duplicate and qualified using the microplate reader (Stat Fax 2100, USA) at 405 and 650nm absorbance. The sensitivity of IFN-γ , IL-10 and TFG-β was 2, 4.1 and 16 pg/mL, respectively.

Statistical Analysis

Data are reported as percentage of the median (95% confidence interval). The Mann–Whitney test was employed for patients versus controls group median comparisons and the Wilcoxon test for serial measurements. A probability (p) value of < 0.05 was considered indicative of significant difference(s).

RESULTS

Clinical Outcomes

Three patients, two from the treatment group and one from the control, discontinued participation and no data from them were included in the analysis. Equally important, we did not find statistically significant difference between baseline EDSS scores and EDSS scores after six months of treatment (Table 1). Furthermore, no related difference between pretreatment and treatment in the average of the mean number of Gd-enhancing lesions was detected during the six-month treatment period (Table 1).

25(OH)D Level

Almost two thirds of all participants had low circulating 25(OH)D levels (18 (64.2%) patients in the treatment group and 21 (61.7%) in the control group. There was no significant difference in the level of 25(OH) D between the two groups at the baseline. Injection of vitamin D_3 (300,000 IU/month) significantly increased the 25(OH) D levels in these subjects after 6 months of treatment whereas no such effects were observed in the control group (Fig. 1).

Table 1: Demographic and clinical characteristics of multiple sclerosis patients at the baseline and 6 months after treatment with vitamin D3

	Treatment group (n = 26)		Control (n = 33)	
	Baseline	6-months	Baseline	6-months
Male/Female	9/17		8/25	
Age(years)(mean(SD))	37 (9)		35 (9)	
Range(years)	18–60		18–60	
EDSS(mean(SD))	2.1 (1.23)	2.31 (1.3)	2.5 (1.12)	2.67 (1.25)
Number of Gd-enhancing lesions(mean(SD))	1.5 (1)	1.9 (0.7)	1.6 (0.8)	2 (1)
Duration of disease(years)(mean(SD))	4.15 (3.3)		6.4 (4.6)	

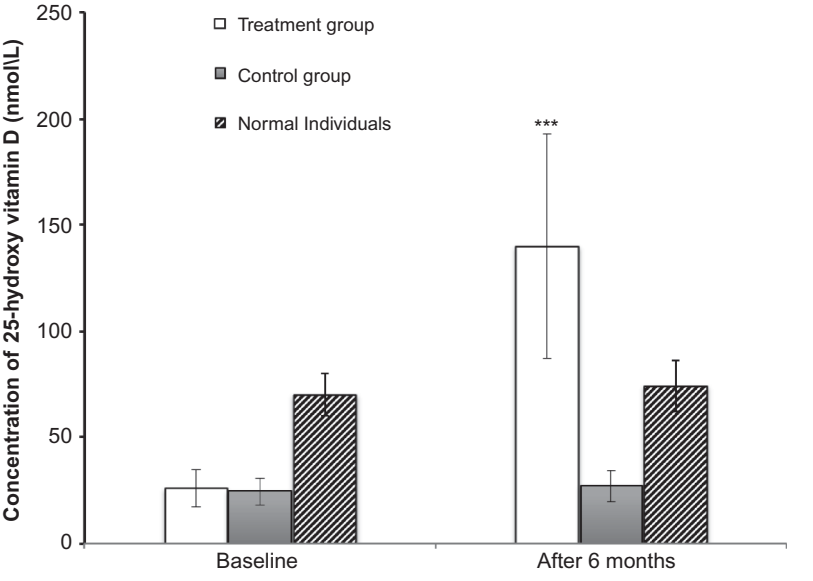

Figure 1: Levels of 25-hydroxyvitamin D in serum of patients with multiple sclerosis before treatment and after 6-month treatment with vitamin D3 (300,000 IU/ month as intramuscular). ***p = 0.0001.

T Cell Function

There were no statistically significant differences in SI between the two groups at the start of study. However, as illustrated by Figure 2, the SI in the vitamin D treatment group after six months of treatment was significantly lower than that in the control group (10.5 ± 1.7 and 16.8 ± 1.3, respectively, p = 0.001, mean ± SD).

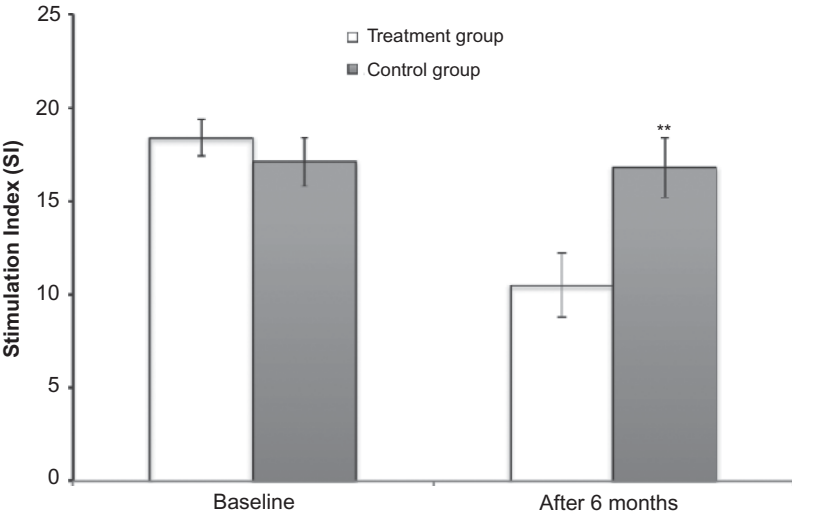

Figure 2: The PHA-induced lymphoproliferation of multiple sclerosis (MS) patients. Peripheral blood mononuclear cells (PBMC) of MS patients cultured in triplicate in the presence of PHA (1 μg/mL). Following 72 h of culture, proliferation was assessed by MTT reduction. The PBMC obtained from Vitamin D treated patients (□) exhibited lower proliferation response to PHA compared with the control group (■) after 6 months' vitamin D treatment.
$^{**}p = 0.001$.

Cytokine Production

There was no significant difference at the baseline in the levels of any of the cytokines between the treated and the control groups (Table 2). Our results indicate that following six months of treatment, the levels of IFN-γ were unaffected by vitamin D. In contrast, the IL-10 level had increased significantly from 3600 ± 430 ρg/mL at the baseline to 4950 ± 530 ρg/mL 6 months later ($p = 0.0001$). Furthermore, the level of TGF-β had increased significantly compared to the baseline level after 6 months of treatment with vitamin D

Table 2: Concentrations of IFN-γ, IL-10 and TGF-β in supernatant of PBMCs of MS patients cultured in the presence of PHA at the baseline and 6 months after vitamin D treatment.

	Treatment group (n = 26)		Control (n = 33)	
	Baseline	6-months	Baseline	6-months
IFN-γ (ρg/ml)	2530 (230)	2250 (143)	2100 (98)	1980 (120)
IL-10 (ρg/ml)	3600 (430)	4950 (530) * **	3430 (312)	3750 (325)
TGF-β (ρg/ml)	2.9 (1.68)	5.2 (2.4) * **	3.1 (1.8)	3.32 (2)

The cytokine levels are given as means (SD). IFN; interferon, IL; interleukin, TGF; tumor growth factor
$^{***}p = 0.0001$.

($p = 0.0001$). Nonetheless, there were no significant differences in IFN-γ, IL-10 and TGF-β concentrations in control group between the baseline and the end of the treatment periods (Table 2).

DISCUSSION

According to the evidence from epidemiological studies, geographic distribution, as well as experimental animal models of MS, indicate a possible influence of vitamin D on disease susceptibility (Ascherio and Munger, 2008; Becklund et al., 2010; Smolders et al., 2008a). There is also some evidence on possible disease-modifying properties of vitamin D in EAE and MS (Cantorna et al., 1996; Fernandes de Abreu et al., 2010; van der Mei et al., 2007).

Several studies have found that oral or intraperitoneal administration of $1,25(OH)_2D$ before EAE-induction (Cantorna et al., 1996; Lemire and Archer, 1991) or after immunization (Muthian et al., 2006; Van Etten et al., 2003) with myelin proteins prevented the appearance of any symptoms. In the animals in which EAE was not prevented, the disease was milder and the disability scores were lower (Nashold et al., 2000) and survival was longer (Branisteanu et al., 1995; Muthian et al., 2006). Moreover, results of some clinical research support the idea that vitamin D treatment is able to modulate immune responses in multiple sclerosis. As an illustration, in 39 MS patients, 6-month supplementation with 25 μg vitamin D led to an increase in plasma 25(OH)D, a significant increase in the cytokine TGF-β and a decline in IL-2 mRNA levels; but correlation of these levels with clinical parameters was not investigated (Mahon et al., 2003).

A study involving 12 patients with supplementation of vitamin D up to 1000 μg/day for 28 weeks showed a decline in the number of Gd-enhancing lesion on MRI per patient (Kimball et al., 2007). In 2 prospective, but very small patient series, supplementation with vitamin D (10 and 125 μg) in combination with other nutriments seemed to have effects on exacerbation rate and EDSS. However, these studies were neither blinded, nor placebo-controlled (Goldberg et al., 1986; Nordvik et al., 2000). In our study, after 6 months of treating MS patients with vitamin D, there were no significant differences in the mean EDSS scores between the treated and non-treated patients. Moreover, no related difference between pretreatment and treatment in the average of the mean number of Gd-enhancing lesions was found during the 6-month treatment period. These differences in results between this study and the abovementioned ones might be explained by differences in route of entry, type and dosage of vitamin D metabolites used, and the genetic backgrounds of the MS patients (Simon et al., 2010).

In this study, we observed a significant reduction in lymphocytes proliferation concomitant to treatment of MS patients with vitamin D. This finding was

evidenced when we compared lymphocytes proliferation after treatment with proliferation at the study baseline and also when we compared lymphocytes proliferation between the treated and the control groups. The molecular mechanisms behind this antiproliferative action have been thoroughly explored (Bouillon et al., 2006). Nevertheless, an in-depth understanding of these mechanisms has not yet been achieved.

There is evidence to support the role of vitamin D as an immune-suppressing agent in this disease. An inhibitory effect on Th1 cell function and a beneficial effect on Th2 and Treg cells have been described *in vitro* (Smolders et al., 2010). The active metabolite of vitamin D (1,25-$(OH)_2$D) has been shown to inhibit *in vitro* T-cell proliferation and production of cytokines, such as IL-2, IL-12, and IFN-γ (Imitola et al., 2005; Lemire et al., 1985; Matheu et al., 2003). Our results revealed that the levels of IFN-γ were unaffected by vitamin D treatment. There is conflicting evidence regarding the effect of vitamin D on IFN-γ production. In EAE, a beneficial effect of 1,25$(OH)_2$D on the clinical and histological features of disease has been described.

Spleen cells from EAE animals produced significantly less IFN-γ after *in vitro* 1,25$(OH)_2$D exposure than before (Muthian et al., 2006). Still this effect was not observed in MBP-specific Th1 lymphocytes (Nashold et al., 2001). In addition, it was found that *in vivo* 1,25$(OH)_2$D did not affect IFN-γ transcription in peripheral lymph nodes (Cantorna et al., 1998).

On the other side, our results showed that the levels of IL-10 and TGF-β had increased significantly after 6 months of treatment with vitamin D ($p = 0.0001$) in comparison with the baseline levels. Mahon et al. (2003) reported that vitamin D supplementation, 1000 IU/day, significantly increased serum TGF-β1 levels in six months. However the TNF-α, IFN-γ, and IL-13 did not differ following vitamin D supplementation (Mahon et al., 2003). The TGF-β1 is produced by regulatory T cells which inhibit the development of EAE and the neutralization of TGF-β1 which increases the severity of the disease (Johns et al., 1991; Thorbecke et al., 2000).

Furthermore, associations have been reported between TGF-β1 and IL-10 levels and appearance of symptoms in humans with MS. Human T cell lines derived from patients with active MS produced less TGF-β1 in comparison with T cells from patients with stable disease (Mokhtarian et al., 1994). Studies have shown that PBMCs have a low level of IL-10 before the onset of disease in relapsing remitting MS patients. The isolated clones during remission showed increased production of IL-10 and TGF-β in the MS patients compared with the controls (Correale et al., 1995; Pelfrey et al., 2000).

In summary, our study demonstrated that injection of MS patients with vitamin D3 (300,000IU/month) for 6 months increased the levels of anti-inflammatory cytokines while at the same patients did not affect the EDSS score of disease or the Th1 immune response. The increased TGF-β1 and IL-10 concentrations following vitamin D supplementation suggest that vitamin D

supplementation may help prevent the development of MS and may be a useful addition to therapy. However, the outcomes of this study were derived from a small samples and can be affected by other variables such as additional vitamin and mineral supplementation. Perhaps a longer period study and/or larger sample size may elucidate some alterations in some or all of these parameters following vitamin D supplementation.

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