The Effect of Vitamin A Supplementation on Disease Progression, Cytokine Levels and Gene Expression in Multiple Sclerotic Patients: Study Protocol for a Randomized Controlled Trial

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Abstract- Multiple Sclerosis (MS) is a chronic inflammatory disease that leads to degeneration of the brain and spinal tissue. Imbalances of CD4+ T cells including Thelper1 (Th1)/Thelper2 (Th2) and Thelper17 (Th17)/Tregulatory (Treg), their secreted cytokines and gene expressions, are important aspects of in immunopathogenesis of MS. Vitamin A and its metabolites can regulate the immune system and appears to be effective in preventing progression of the autoimmune disease such as MS. Disease progression was evaluated By Magnetic Resonance Imaging (MRI), Expanded Disability States Scale (EDSS) and Multiple Sclerosis Functional Composite (MSFC) tests. Cytokine levels were measured using ELISA kits and gene expression was quantified by Real time PCR (RT-PCR) system. According to the difference between the epidemiological and clinical data on the relationship between vitamin A and immune system regulation, this study of the first time assesses Immune function as well as gene expression and progression of the disease following administration of vitamin A supplement.

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Introduction

MS is an autoimmune and neurodegenerative disease of the central nervous system (1). Inflammation in MS leads to both demyelination and axonal loss. Injury from the disease may cause brain atrophy that can be measured by MRI. Atrophy of the central nervous system occurs at the onset of the disease and progresses throughout its advanced stages. Although atrophy correlates only modestly with current clinical disability, the progression of atrophy more strongly predicts progression of later disability (2). The correlation between damage to the central nervous system and inflammatory reactions during progression of the disease can be understood in terms of existing myelin specific autoreactive T cells in the peripheral blood and cerebrospinal fluid (CSF) of MS patients (3). This "activated state" of myelin-reactive T cells observed in MS patients is associated with up-regulation of adhesion molecules that make these cells more prone to interact with the blood–brain barrier (BBB) and drive an inflammatory response directed against myelin antigens

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within the CNS (4). Therefore, any change in immune response induced by CD4+ cells, particularly the imbalance between Th1/Th2 and also Th17/Treg and subsequent changes in their cytokine's secretion such as IFN-γ, IL-2(Th1), IL-4 (Th2), IL-17(Th17) IL-10 and TGF-β (Treg) can lead to damage and atrophy of the brain (5,6). In addition to immunological changes, there is a clinical-radiological paradox in MS, in that MRI findings do not always accurately reflect the patient's clinical symptoms. Therefore, a combination of MRI and immunologic evaluation in a clinical study may be more valuable (7). This is particularly relevant to MS patients that incur an imbalance of cytokines such as increasing Th1 immune response and decreased Th2 and Treg function. Cytokine changes in MS are characterized by increased levels of IFN-γ, IL-2, and IL-17 and decreased levels of IL-4, IL-10 and TGF-β (8).

CD4+ Treg cells can suppress inflammatory responses and induction or enhancement of creation of their cytokines such as TGF-β represents a potentially interesting option for the treatment of MS (9). Therefore, therapeutic strategies that lead to shift the immune response reactions from Th1 to Th2 and Th17 to Treg cells may be effective in the treatment of MS (10). Retinoic acid inhibits Th1 and Th17 polarization and induces the differentiation of Th2 and Treg cells (11, 12). Other research has demonstrated that all Trans' retinoic acid (ATRA) causes a shift in gene expression of Th1, and Th17 cytokines and their transcription factors to Th2 and T regulatory cells (13). This study examines the effect of vitamin A supplementation as synergistic effect on MRI changes, cytokine levels and gene expression in patients with MS.

Materials and Methods

Study Design

This study is a randomized double blind placebo controlled phase III clinical trial. Phase I of this study is the evaluation of MRI changes of patients. Phase II is measurement of cytokine levels in serum and soup of cultured cells and phase III include gene expression by RT-PCR device.

Susceptibility testing

All the drugs were purchased from Sigma Chemical (St. Louis, MO). The drug-susceptibility testing against isoniazid (0.2μg/ml), streptomycin (4μg/ml), pyrazinamide (2μg/ml), ethambutol (2μg/ml), ethionamide (2μg/ml), rifampicin (40μg/ml), kanamycin (20μg/ml) were performed according to the CDC standard conventional proportional method (10), on slants using H37Rv strain as a positive control. The drug-susceptibility testing against second-line drugs kanamycin (20 μg/ml), amikacin, capreomycin(10 μg/ml),ciprofloxacin(2 μg/ml), cycloserine, ethionamide (20 μg/ml), and para-aminosalicylic acid was performed using 2 critical proportions of 1%.

Participants

Patients include one hundred and one subjects (77 women and 24men) aged 20-45 years old. They are identified by a neurologist based on Mc Donald criteria that have relapse remitting MS and in the current period of remission. Patients are selected for the study based on the specific inclusion and exclusion criteria, as described below. One hundred and three patients are selected from the Imam Khomeini Hospital the (Tehran-Islamic Republic of Iran). The complete Blood Count (CBC), liver enzymes (e.g. aspartate aminotransferase and alanine aminotransferase) activity and the plasma levels of cholesterol and triglyceride (TG) are measured before starting supplementation. These tests are done routinely every 3 months when referring patients to

Hospital during interferon beta 1a consumption, and it is not necessary to allocate costs or separate tests for patients. Patients are excluded if they have abnormal biochemical test results. Then those who meet the inclusion criteria and who are at the same phase of the illness take the same drug (interferon beta 1a). Patients are randomized on the basis of age and gender by blocking; the patients are divided into two groups so that there are no differences between the two variables of age and gender. The intervention group undergoes retinyl palmitate supplementation of capsules containing25000 IU daily for 6 months and 10000 IU/day for another 6 months. The control group, as well as the intervention group, are administered the placebo supplement in the same way. The content of placebo capsules is edible paraffin oil.

Ethics

The patients are informed about the aim and possible risks of the study and are free to leave the study at any time. Written informed consent of the Tehran University of Medical sciences is obtained from all of the participants at the start of the trial. The study is approved by the Ethics Committee of the Tehran University of Medical Sciences (ID: 8887, 9567, 10033) and registered according to the appropriate clinical trial registration system (NCT01407211, NCT01225289 and NCT01417273).

Inclusion Criteria

1) Definitive diagnosis of the disease based on Mc Donald criteria
2) Clinical course of relapse-remittance
3) Age 20-40 years (pre-menopause)
4) EDSS between 0-5
5) Voluntary cooperation
6) Use of interferon beta 1a (CINOVEX® or AVONEX®) weekly, for three months before participating in the study

Exclusion Criteria

1) Fulminant symptoms of disease such as hepatitis or pancreatitis
2) Use of multivitamin supplements three months prior to participation in the study
3) Evidence of any disease effective on the Th1/Th2 and Th17/Treg balance such as asthma, rheumatoid arthritis, Type 1 diabetes and inflammatory bowel disease.
4) Abnormality of the blood and liver function test.
5) Malnutrition and BMI<18.5
6) A history of cardiac infarction and brain stroke
7) Pregnancy
8) A history of alcohol abuse
9) Increasing EDSS of more than one during the duration of the study
10) Evidence of more than one remitting attack during the study
11) Any allergic reaction to vitamin A during the study
12) Patients with CIS and neuromyelitis optica were not included (14, 15).

Questionnaire and Functional Tests

At the start of the study, each patient is presented with a set of questionnaires; a general information questionnaire, a questionnaire to record 3 daily recalls andfood frequency questionnaire (FFQ), one to be completed at the start of taking supplements and another for the end of the period of taking supplements. Weight and height are measured at the beginning and end of the trial. Clinical examination is done for all patients at the start of the study and every three months and at the end of supplementation. EDSS scores are calculated by a neurologist. MSFCs tests are performed on all patients at the beginning and at the end of the study (EDSS and MSFC tests are a very useful means for assessment of progression of the disease especially if they are combined by MRI evaluation).

MRI protocol

Image Acquisition

All patients are subjected to a baseline brain MRI and a one-year follow up scan at the end of the trial. Imaging is performed using a GE Signa 1.5T MRI scanner (General Electric Medical Systems, Milwaukee, WI). Whole-brain MRI protocol at the baseline consists of: (i) axial high-resolution T1-weighted 3D IR-SPGR (TR/TE = 13/4 ms, TI= 400ms, slice thickness= 1.2 mm, gap= 0 mm, matrix size= 512×512, in-plane pixel spacing= 0.5×0.5 mm, flip angle= 30°, FOV= 26 cm, number of averages= 1); (ii) axial T2-weighted FSE (TR/TE = 6000/100 ms, slice thickness= 1.5 mm, gap= 0 mm, matrix size= 512×512, in-plane pixel spacing= 0.5×0.5 mm, FOV= 24 cm); (iii) axial PD-weighted FSE (TR/TE = 3000/20 ms, slice thickness= 1.5 mm, gap= 0 mm, matrix size= 512×512, in-plane pixel spacing= 0.5×0.5 mm, FOV=24 cm). An intravenous dose of gadoteric acid (Dotarem®, Guerbet, France) (0.1 mmol/kg body weight) is administered during the follow up scan, and a post-gadolinium (post-Gd) T1-weighted FSE axial image (TR/TE = 660/20 ms, slice thickness= 1.5 mm, gap= 0 mm, matrix size= 512×512, in-plane pixel spacing= 0.5×0.5 mm, FOV= 24 cm) is obtained in addition to the baseline protocol with a scan delay of 10 minutes. Total acquisition time is approximately 30 minutes at baseline, extended to 40 minutes at the follow up (16).

Image Analysis

Preprocessing is performed using FSL (FMRIB's Software Library, www.fmrib.ox.ac.uk/fsl) (16). High-resolution T1-weighted images are resampled to isotropic 2 mm voxels using a science-based interpolation algorithm. Each patient's T2- and PD-weighted and post-Gd images are registered to the corresponding T1-weighted scan using a 6-parameter rigid body transformation implemented in FLIRT (FMRIB's Linear Image Registration Tool, part of FSL, www.fmrib.ox.ac.uk/fsl/flirt) (17), and then undergo noise reduction using SUSAN (Smallest Univalue Segment Assimilating Nucleus, part of FSL, http://fsl.fmrib.ox.ac.uk/fsl/susan) (18). Lesions are segmented from T2-weighted and post-Gd images using a semi-automatic manual tracing pipeline available in Amira® (Visage Imaging GmbH, www.amira.com), with reference to the co-registered PD-weighted images. Percentage change of brain volume between the baseline and the follow up is estimated from high-resolution T1-

weighted scans with SIENA (Structural Image Evaluation, using Normalization, of Atrophy, part of FSL, www.fmrib.ox.ac.uk/fsl/siena) (19).

Sample blood collection and Peripheral Blood Mononuclear Cell (PBMC) separation

After performing MRI, seventy-five relapsing–remitting MS patients consented to participate in the second phase of this survey (written consent was obtained from all of them); four patients withdrew from the study for changing drug and personal reasons mid-project so that 71 people remained until the end of second phase of the study. Blood samples were collected from subjects in sterile tubes with heparin as an anticoagulant. One ml of whole blood samples was collected in a sterile microtube without any anticoagulant for serum separation. PBMCs were isolated using the Ficoll-Histoprep gradient (BAG Health Care GmbH, Germany) centrifugation protocol. Cells were counted and diluted to 1×106 cells per ml culture medium (RPMI 1640; Gibco, Invitrogen, UK) supplemented with 10% heat inactivated fetal calf serum (Gibco, Invitrogen, UK) and 100U/ml penicillin/streptomycin (Sigma, USA) (20, 21).

Cell proliferation assay

Proliferation assay was performed using colorimetric bromodeoxyuridine (BrdU) test (Roche, Basel, Switzerland).

After dilution of cells and preparing 1×106 cells per ml consternation, 500 µl of the diluted cell suspension was separated, and 10% fetal calf serum (FCS; Gibco, Invitrogen, UK) was added to the remaining cells. 75000 PBMCs were cultured in 96-well flat-bottomed microtiter plates (Nunc, Thermo Fisher Scientific Inc, Denmark) in the presence of 5 µl/ml Myelin Oligodendrocyte Glycoprotein (MOG) [(35-55) human; Anaspec, USA]. 75000 PBMCs from diluted cells without FCS were cultured in the presence of MOG and MOG as well as 10 µl of serum from patient's blood cells. 75000 cells were left stimulated with 5 µl/ml phytohemagglutinin (PHA; Sigma, USA) and untreated Cells were incubated at 37ºC in a 5% CO2 incubator for 96 hours. After this period, cell proliferation ELISA BrdU kit (Roche Diagnostics GmbH, Germany) was used for cell proliferation assessment. Briefly 15 µl/well of BrdU labeling solution was added and re-incubated the cells for additional 4 hours at 37°C, and then plate was centrifuged at 300×g for 10 minutes. The supernatant was discarded by flicking off, and then cells were dried by hair-dryer airflow. 200µl FixDenat was added to all wells and incubated at room temperature for 30 minutes. Following incubation, FixDenat solution was removed thoroughly by flicking off and tapping. 100µl/well anti-BrdU-POD working solution was added and incubated for 90 minutes at room temperature. Antibody conjugate was removed by flicking off and rinsing wells three times with 200µl/well washing solution (PBS, 1×) (21).

Measurement of Cytokine Levels

Cytokine levels were measured in Plasma and cell culture soup obtained from thirty five patients randomly. For this purpose separated plasma was used, and cells were cultured in a 24-well flat-bottomed plate. In this step, 750,000 cells were cultured in six wells in the presence of MOG, PHA and three wells remained untreated Cells were incubated at 37ºC in a 5% CO2 incubator for 96 hours after this period culture soup of these cells were collected and aliquoted in microtubes to determine cytokine concentrations.IL2, IL4, IL10, IL17, TNF-α and TGF-β levels in plasma and soup of cultured cells were measured by ELISA kit (eBioscience USA)

mRNA and cDNA synthesis

Thirty six patients were randomly selected for evaluation of gene expression. For this purpose, 4x106 PBMCs used freshly for RNA extraction. In this step cells were cultured as explained above and after 96 hours' incubation RNA was synthesized from proliferated cells in the presence of MOG, PHA and untreated cells by RNeasy Mini Kit (Qiagene-USA). Purification of RNA was confirmed by Nanodrops. After this, cDNA was synthesized from RNA by a QuantiTect Rev. Transcription Kit (Qiagene-USA). Purity of extracted RNA was checked by Nanodrop spectrophotometer (NanoDrop Technologies, USA). A ratio of A 260/280 ranging 1.9 to 2.1 was considered as acceptable (21).

RT-PCR

Gene expression of IL4, IL17, IFN-γ and TGF-β transcription factors related to these genes including GATA 3, ROR-C, T-bet, and FOXP3, will be quantified by RT-PCR. RARα and RARγ will be measured using RT-PCR too .A primer for these genes was designed by Primer express 3 software (Applied Biosystem, Foster city, CA, USA) and prepared by Metabion and Bioneer Company (Table 1). Power SYBR Green PCR Master Mix (Applied Biosystems-USA) is used for the reactions (this step will be done in the following months) (Table 1). PCR will process in special optical tubes in 48- reaction

plates (MicroAmp Optical, ABI) with 20 µl reaction mixture including 10µl Power SYBR® Green PCR Master Mix (Applied Biosystem, Foster city, CA, USA), 7µl DEPC treated water, 0.5µl forward primer, 0.5µl reverse primer and 2µl cDNA as template. The wells were sealed with optical adhesive film (Applied Biosystem, Foster city, CA, USA), and the plate was centrifuged for several seconds at high speed.

Amplification conditions were performed using the standard two-step run protocol; step 1:10s at 95°C, step 2: 40 cycles of 15s at 95°C plus 1s at 60°C. After completion of amplification cycles, melt curve was generated to verify if a single gene product had been amplified. For each gene, mRNA expression level was normalized to the level of β-actin as house-keeping gene (21).

Table 1. Sequencing and information about primers

Gene Name	Sequence	Length	Tm	CG%
IL-4 (F)	CTGCAAATCGACACCTATTAATGG	24	52.44	41.67
IL-4 (R)	GCACATGCTAGCAGGAAGAACA	22	55.34	50.00
IL-10 (F)	CCAGGGCACCCAGTCTGA	18	55.03	66.67
IL-10 (R)	TCGGAGATCTCGAAGCATGTT	21	53.42	47.62
IL-17 (F)	GGGCCTGGCTTCTGTCTGA	19	55.70	63.16
IL-17 (R)	AAGTTCGTTCTGCCCCATCA	20	53.71	50.00
T-bet (F)	TGCTCCAGTCCCTCCATAAGTAC	23	55.18	52.17
T-bet (R)	TCTGGCTCTCCGTCGTTCAC	20	56.11	60.00
GATA3 (F)	AGATGGCACGGGACACTACCT	21	56.97	57.14
GATA3 (R)	CCTTCGCTTGGGCTTAATGA	20	52.46	50.00
TGF-beta (F)	CTCTCCGACCTGCCACAGA	19	55.16	63.16
TGF-beta (R)	AACCTAGATGGGCGCGATCT	20	55.22	55.00
RORc (F)	GAAGTGGTGCTGGTTAGGATGTG	23	55.54	52.17
RORc (R)	GCCACCGTATTTGCCTTCAA	20	53.35	50.00
Foxp3 (F)	GCAAAGTTGTTTTTGATACGTGACA	25	53.68	36.00
Foxp3 (R)	AGGCTTGGTGAAGTGGACTGA	21	55.32	52.38
RARα(F)	CTGCCAGTACTGCCGACTGC	20	52.23	56.00
RARβ(R)	ACGTTGTTCTGAGCTGTTGTTCGTA	25	54.12	55.00
RARγ(F)	TCCTAAAGCATACGGGTCCTGGCAT	--	53.26	43.00
RARγ(R)	CGCTCCATGGCCTCCACAATATTCA	--	55.78	39.00
Beta-actin (F)	CCTGGCACCCAGCACAAT	18	54.46	61.11
Beta-actin (R)	GCCGATCCACACGGAGTACT	20	55.87	60.00

RBP/TTR index measurement

The molar ratio of retinol-binding protein to transthyretin (RBP/TTR index) has been proposed as an indirect method to assess the vitamin A status in children with inflammation. RBP/TTR index is measured by highly sensitive ELISA kits (Icl lab USA) as previously described (22).

Statistical methods

Data are expressed as mean ± SD and are analyzed by SPSS software (version 18.5). For compression between, before and after variables and within groups paired t-test and student sample t-test are used respectively. The test level for statistical significance of differences between both treatment arms is defined as $p \leq 0.05$ for all tests.

Results

This clinical trial study, a collaboration between the School of Public Health and Iranian Center for Neurological Research, has been funded by Tehran University of Medical Sciences and Health Services grants (ID: 8887, 9567, 10033).

Discussion

Inflammation and degeneration of the central nervous system is an important cause of demyelination in MS. Many Immunological studies have been done on experimental autoimmune encephalomyelitis (EAE) as an animal model for MS-like diseases (23). Initially, IFN-γ-producing Th1 cells and more recently, IL-17-producing Th17 cells with specificity for myelin antigens have been implicated in EAE induction (24). Based on the results obtained from studies on this model and observations obtained from studies in MS patients, changes of some gene expression and subsequently cytokine levels and degeneration of brain tissue should be investigated.

On the website of the National MS Society, there are more than 136 ongoing clinical trials trying different

treatments for multiple sclerosis. There are currently 8 FDA approved agents for cure of relapsing forms of MS (25). This indicates that a new approach for the treatment of the disease will be essential and effective.

Vitamin A and its metabolites such as retinoic acid (RA) regulate immune homeostasis by induction of regulatory T cells. Studies have shown that RA also elicits pro-inflammatory Th1 and Th17 cells' responses to infection. Retinoic acid receptor alpha (RARα) is a critical mediator for these effects. These findings demonstrate a functional role for the RA-RARα axis in the development of both regulatory and inflammatory reactions of adaptive immune system (26).

Vitamin A deficiency is associated with decreased Th2 responses. Vitamin A supplement inhibits Th1 and promotes Th2 differentiation in vitro and *In vivo*. RA promotes Th2 differentiation by inducing Gata3, Stat6, and IL-4 genes, and inhibits T-bet expression. RA also has an indirect effect on Th2-promotion by modulation of dendritic cell (27). Therefore, retinol and its metabolites present promising possibilities to prevent inflammatory reactions, central nervous system degeneration and disease progression. Retinyl palmitate could decrease PBMCs proliferation in the presence of MOG (28). It seems that the retinyl palmitate, after converting to retinoic acid, binds to RAR receptors, resulting in the modification of cytokine gene expression and cell proliferation (29).

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