

Interferon β -1a and Atorvastatin in the Treatment of Multiple Sclerosis

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ABSTRACT

Background: Statins, widely used cholesterol-lowering agents, have also been demonstrated to have anti-inflammatory and immunomodulatory effects. **Objective:** To evaluate the effects of atorvastatin in combination with Interferon- β in the treatment of multiple sclerosis (MS) in a randomized controlled clinical trial. **Methods:** Multiple sclerosis patients were randomized independently, in a double blind design, into one of two treatment groups. Control group (n=45) received 30 μ g/week interferon β -1a via intra-muscular injection. Atorvastatin-treated group (n=50) received interferon β -1a similar to control group in addition to atorvastatin (40 mg/day) for 18-months. All clinical and immunological variables were measured at the baseline and at the end of the study. **Results:** There was no significant difference between the two groups in the expanded disability status scale scores and the number of gadolinium-enhancing lesions during the 18-month treatment period. After 18 months, the levels of interleukin (IL)-4, IL-10, transforming growth factor- β and serum ferric reducing antioxidant power in the atorvastatin treatment group were significantly higher than the control group. Levels of IL-17, TNF- α and lymphocyte proliferation in the atorvastatin treatment group were significantly lower than the control group. **Conclusion:** Although combined atorvastatin and interferon- β do not change the clinical course of MS, atorvastatin might have beneficial effects in MS treatment possibly through inducing anti-inflammatory responses.

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Keywords: Atorvastatin, Cytokines, Expanded Disability Status Scale Scores (EDSS), Interferon β -1a, Multiple Sclerosis, Nitric oxide

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INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system that is thought to be mediated by TH17 and TH1 immune responses (1). Pathological studies reveal that different effector mechanisms such as dis-regulation of immune responses and free radicals are involved in the inflammation, demyelination, and tissue damage observed in MS patients (2).

Interferon- β is a standard drug for treatment of MS (3). It reduces disease activity by altering peripheral and CNS immune responses (4). Because of heterogeneous pathology of MS and complex immune dis-regulation and neurodegenerative processes, some MS patients do not respond or have minimal response to interferon- β therapy. Therefore, combination of interferon- β with other immunomodulating or anti-inflammatory agents may be more effective in treatment of MS.

Statins are inhibitors of 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase, which are widely prescribed for their cholesterol-lowering properties in order to reduce atherosclerosis and cardiovascular morbidity (5). Over decades, statins have established themselves as generally safe and well-tolerated drugs. Recent studies in animal models of autoimmune diseases demonstrate anti-inflammatory and immunomodulatory properties of statins that might be beneficial in the treatment of neurodegenerative diseases (6-8).

In experimental autoimmune encephalomyelitis (EAE) models, atorvastatin has been shown to promote differentiation and expansion of myelin protein-reactive regulatory TH2 cells and suppress the expression of MHC class-II and costimulatory molecules on antigen presenting cells (9-11).

A previous pilot study with oral simvastatin given daily over 6 months showed a significant reduction of contrast-enhancing lesions (CEL) in brain magnetic resonance imaging (MRI) of 30 RRMS patients compared to a 3 month baseline period (12).

Safety studies on RRMS patients showed that oral high-dose atorvastatin (80 mg/day) is safe and well tolerated for a 9-month period. Moreover, MRI analysis indicated a possible beneficial effect of atorvastatin, alone or in combination with IFN- β -1a, on the development of new CELs (13). However, to date, it is not well clear whether statins have immunomodulatory effects in MS. In this clinical trial study, we investigated the effect of IFN- β -1a and atorvastatin as add-on treatment on the immunological and neurological variables in relapsing–remitting MS (RRMS) patients.

MATERIALS AND METHODS

Trial Design. Patients aged 18 to 60 years from hospitals of Medical University of Arak, were randomized independently into two groups (Figure 1). Control group (n=45) received 30 μ g/week interferon β -1a (CinnovexTM, Fraunhofer Institute, Germany) as intra-muscular injection. Atorvastatin-treated group (n=50) received interferon β -1a as control group with atorvastatin (40 mg/day) for 18-months (trial code: IRCT2015112925284N1). All clinical and immunological variables were measured at the baseline.

Participants. Patients were diagnosed with MS by the revised 2010 McDonald criteria (14). The patients were recruited from December 2012 to June 2014. Inclusion criteria

were confirmation of relapsing-remitting MS in patients 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, drug abuse; renal failure; pregnancy, hepatitis diseases. Besides during the study, data of MS patients with acute clinical relapses occurring during the study were considered as drop-out (Figure 1).

The study was performed in keeping with the Helsinki declaration on research with human subjects, and the protocol approved by the local ethics committee (Ref. Number: AUMSec-88-69-1). Before providing informed written consent, all patients were advised of the approved alternative therapies available to them.

Interventions.

1. Neurologic Evaluation. Physical and neurological examinations (EDSS) were performed every 6 months. The MRI with gadolinium was performed in the beginning of the study and at the end.

2. Immunological Examinations. All para-clinical and immunological variables were assessed at the baseline and at the end of the study. 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% heatinactivated 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 (15).

3. Proliferation of PBMC. Proliferation was evaluated by the MTT assay method. A total of 3×10^3 cells in 200 µl RPMI-1640 supplemented with 10% FBS were stimulated with 1 µg/mL PHA. The plates were then incubated in a 5% CO₂ at 37°C for 72 h. Twenty microliters of 5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl; Sigma-Aldrich, St. Louis, MO) were added to the cells, followed by incubation for 4h. After centrifugation, the medium was removed, and 200 µl 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 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 (15).

4. Cytokines Analysis. For cytokine detection, PBMCs at a density of 2×10^6 cells/mL were incubated in 1mL cultures once in presence, and once in absence, of PHA (1 µg/mL) and cultured in cell culture condition for 72h. The supernatants were collected and interferon gamma (IFN-γ), interleukin (IL)-4, IL-10, IL-17, transforming growth factor-β (TGF-β) and tumor necrosis factor (TNF)-α 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 450 and 650nm absorbance. The sensitivity of IFN-γ, IL-4, IL-10, IL-17, TNF-α and TGF-β were 8, 4.46, 0.3, 1.1, 1.5 and 16 pg/mL, respectively.

5. Nitric Oxide Determination. Nitric oxide (NO) was assayed by measuring nitrite as the end product of reaction, which was determined by a colorimeter assay based on the Griess reaction. Griess reagent was prepared by solving 1g sulfanilamide in 100 mL phosphoric acid 5% mixed with 0.1 g naphthyl ethylene diamineHCl (NED) in 100 mL distilled water. Serum sample (100 μ L) was mixed with 100 μ L of Griess reagent at room temperature for 10 min. Absorbance was measured using ELISA plate reader (Stat fax2100, USA) at 550 nm. Concentration of nitrite was determined by standard curve of sodium nitrite 0.1 molar prepared in distilled water.

6. FRAP Measurement. Total antioxidant capacity (TAC) was measured according to the Ferric Reducing/Antioxidant Power (FRAP) test. In the FRAP test antioxidants in the sample reduce ferric tripyridyltriazine (FeIII-TPTZ) complex, to a blue colored ferrous tripyridyltriazine (FeII-TPTZ) form, with an increase in absorbance at 593 nm. Briefly, the working FRAP reagent was prepared by mixing 300 mM acetate buffer (pH=3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution (Sigma- Aldrich, St. Louis, MO, USA) in 40 mM HCl, and 20 mM FeCl₃•6H₂O in a 10:1:1 ratio just before use and heated to 37°C. The 300 μ L reagent and the 10 μ L standard (FeSO₄•7H₂O) or plasma sample were added to each well in a 96 well (300 μ L) micro titer plate and mixed well. The absorbance readings were taken at 593 nm immediately after and 4 min after using an ELISA plate reader (Stat fax2100, USA). The plate was incubated at 37°C for the duration of the reaction. All standards and samples were run in triplicate. Standards of known Fe (II) concentrations (FeSO₄•7H₂O) were run using several concentrations between 50-1000 μ M. A standard curve was then prepared by plotting the concentration for each standard versus its absorbance.

Outcomes. Total antioxidant capacity, NO and Cytokines analysis (IFN- γ , IL-4, IL-10, IL-17, TNF- α , and TGF- β) were the primary endpoints and physical and neurological examinations were the secondary endpoints.

Sample Size. The sample size was calculated by Pocock method for RCTs and 45 in each group was found appropriate and due to the probability of drop-out we increased it to 50 for each group.

In this RCT 32 patients were dropped out due to the lack of regular consumption of the medicines, lack of attendance for laboratory tests, 3 cases for adverse effects of the intervention (2 cases due to abdominal pain after Atorvastatin administration and one case due to CPK and LDH increase)

Randomization. The subjects with random table were randomly assigned to the groups when they were eligible to be entered the study without any factor or characteristics to be considered for being on any particular group.

Blinding. The patients were not aware of the study group in which they were assigned; moreover, the neurologist, the physician and the radiologist were unaware of the treatment assigned to each patient. Staff members performing the magnetic resonance imaging (MRI) were blinded for the clinical course, and physicians assessing the neurological status of the patients were blinded for the MRI results.

Statistical Methods. SPSS version 16 was used for all statistical analyses. Data are presented as mean \pm SEM. For clinical scores, significance between groups was examined by Mann-Whitney *U* test. All other statistical comparisons between groups were examined using 1-way multiple range ANOVA test for multiple comparisons. *P* values less than 0.05 were considered statistically significant.

RESULTS

Clinical Outcomes. In this trial, 95 patients were enrolled of which 50 were in case (mean age; 39 ± 10) group and 45 were in control (mean age; 36 ± 14) group; of which 80 (84.2%) were female. There were no significant differences between the groups regarding age and gender (Table 1).

Table 1. Demographic and clinical characteristics of multiple sclerosis patients at the baseline and 18 months after treatment with atorvastatin.

	Treatment group (n=50)		Control (n=45)		P Value
	Baseline	18-months	Baseline	18-months	
Male/Female	6/44		9/36		
Age(years)[mean(SD)]	39(10)		36(14)		N.S*
Range(years)	18-60		18-60		
EDSS[mean(SD)]	2.6 (1.2)	3.1 (1.3)	2.8 (1.6)	3.3 (1.6)	N.S
Number of Gd-enhancing lesions[mean(SD)]	1.5 (1)	1.9 (0.7)	1.6 (0.8)	2 (1)	N.S
Duration of disease(years)[mean(SD)]	4.1(1.9)		4.6(2.3)		N.S

*N.S: Not significant

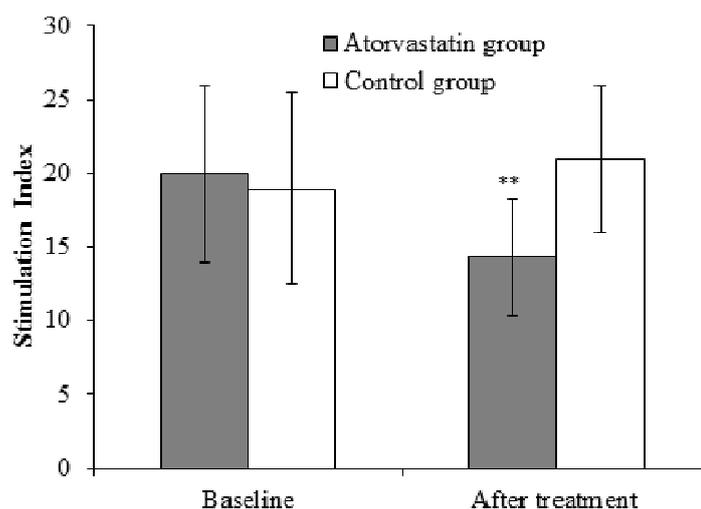
At the baseline, there was no significant difference between the two groups in the levels of immunological and neurological variables (Tables 1 and 2). We did not find statistically significant differences between baseline EDSS scores and EDSS scores after 18 months of treatment (Table 1). Furthermore, no related difference between pretreatment and treatment in the average of the mean number of Gd-enhancing lesions was detected during the 18-month treatment period (Table 1).

Cytokine Production. There were no significant differences at the baseline in the levels of any of the cytokines between the treated and the control groups (Table 2). Our results indicated that following 18 months of treatment with atorvastatin, the level of IFN- γ was unaffected. In contrast, the levels of IL-10, TGF- β and IL-4 increased significantly after atorvastatin treatment ($p=0.0001$). Furthermore, the levels of TNF- α and IL-17 decreased significantly compared to the baseline level after 18 months of treatment with atorvastatin ($p=0.0001$), (Table 2).

Table 2. The levels of IFN- γ , TNF- α , TGF- β , IL-4, IL-10 and IL-17 in the supernatant of PBMCs of MS patients cultured in the presence of PHA at the baseline and 18 months after atorvastatin treatment.

Cytokines	Treatment Group (n=50)		P Value	Control (n=45)		P Value
	Baseline	18-months		Baseline	18-months	
IFN- γ (pg/ml)	1470(498) *	1600(367)	NS**	1580 (547)	1770(620)	NS
IL-10 (pg/ml)	550(420)	1050(360)	0.0001	760(310)	695(504)	NS
TGF- β (pg/ml)	5.9(1.68)	11.3(2.4)	0.0001	4.1(1.8)	6.32(2)	NS
IL-4(pg/ml)	12.8(6.2)	25.3(4.2)	0.0001	13.1(3.2)	12.7(4.1)	NS
IL-17(pg/ml)	270(74)	125(67)	0.0001	198(95)	220(86)	NS
TNF- α (pg/ml)	1420(1220)	960(720)	0.0001	1610(810)	1850(890)	NS

Lymphocyte Proliferation. There was no difference in the stimulation index (SI) at the baseline between the two groups. However, the SI in the atorvastatin treatment group after 18 months of treatment was significantly lower than that of the control group (13.5 ± 4.8 and 18.3 ± 6.4 , respectively, $p=0.001$), (Figure 2).

**Figure 2.** Cell proliferation assay. Peripheral blood mononuclear cells (PBMC) of MS patients cultured in triplicate in the presence of PHA (1 μ g/mL). Following 72h of culture, proliferation was assessed by MTT reduction. Data were presented as mean \pm standard deviation. **: $p=0.001$

Nitric Oxide Level. The concentration of NO was determined with the Griess reaction, as described previously. The serum level of NO was not significantly different in the atorvastatin treatment group when compared with the control group at the baseline and 18 months after treatment (Table 3).

Table 3. Concentration of nitric oxide (μM) in the sera of multiple sclerosis patients before and 18-months after treatment with atorvastatin.

Groups	Baseline	After 18 months	P. Value
Atorvastatin group	$[11.45 \pm 4.7]^*$	10 ± 5.4	NS
Control group	14.3 ± 10.7	16.56 ± 13	NS
P Value	NS	NS	

Antioxidant Capacity. FRAP reaction was performed on plasma sample. Results showed a significant increase of mean serum value of FRAP in atorvastatin treatment group ($410 \pm 23 \mu\text{mol}$) compared to those in the control group ($300 \pm 35 \mu\text{mol}$) at the end of the study ($p = 0.001$). Data are presented in Figure 3.

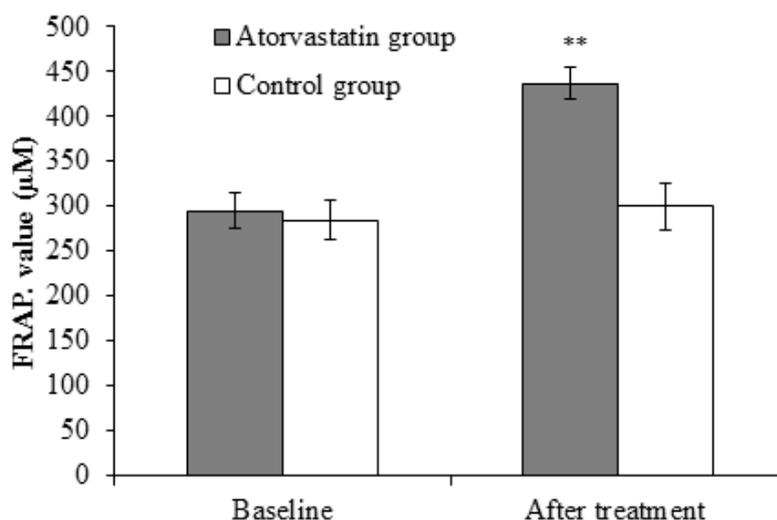


Figure 3. Antioxidant capacity was measured by Ferric Reducing/Antioxidant Power (FRAP) reaction. Data are presented as the mean (SD). **, $p=0.001$.

DISCUSSION

Several clinical studies showed that statins in combination with interferon- β have different effects on clinical score and disease activity in MS patients. Our results showed that there is 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 18-month treatment period. Recently, Kamm and colleagues in a randomized controlled trial study showed that atorvastatin 40 mg/day in addition to interferon β -1b did not have significant effect on the total number of new Gd-enhancing lesions, volume of grey and white matter, EDSS and relapse rate compared to interferon β -1b monotherapy (16). Also, a post-hoc analysis study on 40 patients with relapsing-remitting multiple sclerosis showed that combination of IFN β -1a (30 μ g/week, intra-muscular) and atorvastatin did not change clinical activity, CELs, or T2-lesions (17). Also, the results of our study are similar to the results of the SIMCOMBIN trial that combination of simvastatin (80 mg/day) and IFN β -1a (30 μ g/week) did not change the clinical activity of the disease in MS patients (18).

The results of other clinical studies showed that statin added-on IFN β -1a reduced or inhibited disease activity. Vollmer and co-workers observed that oral simvastatin (80 mg/day) significantly declined the number and the volume of Gd-enhancing lesions in RRMS patients (12). In an open-label study, addition of high-dose atorvastatin (80 mg/day) to IFN β -1a therapy reduced Gd-enhancing lesions in RRMS patients (13). Simvastatin added on to intramuscular IFN β -1a reduced the total relapse rate, significantly (19). Low-dose atorvastatin (20 μ g/day) added on subcutaneous IFN β -1a reduced Gd-enhancing lesions and relapses in MS patients (20). Besides, some animal models and clinical studies showed that statins have a negative effect on the disease course in EAE and MS (21-23). For instance, a double-blind, placebo-controlled trial study found that combination of atorvastatin (80 mg/day or 40 mg/day) to IFN β -1a increased MRI and clinical disease activity (22).

Multiple sclerosis is a TH1 cell-mediated chronic inflammatory disease. Many immune cells such as dendritic cells, monocytes, TH17 cells and B-cells are also involved in the immunopathology of MS (24). These cells produce free radicals, nitric oxide, inflammatory cytokines and antibodies that lead to damage of myelin in the central nervous system. TH2 cells and Treg cells secrete anti-inflammatory cytokines and TGF- β that suppress the immune response. Higher levels of IL-17, IFN- γ and TNF- α are found in the serum of MS patients. However, immunomodulation of the immune responses and shift of TH1-mediated immune response to TH2 may have positive impact on the treatment of MS.

The initial studies on animal models of MS showed that statins have immunomodulatory and anti-inflammatory functions in addition to cholesterol-reducing effect (9, 25, 26). Youssef and colleagues showed that oral atorvastatin prevented the development of chronic, relapsing paralysis in EAE. Atorvastatin was also found to induce phosphorylation of STAT6 that induces TH2 cytokines secretion, and inhibit phosphorylation of STAT4 that induces TH1 cytokines secretion. Therefore, atorvastatin treatment caused a shift from proinflammatory TH1 profile to an anti-inflammatory TH2 profile (9,11,27).

In our clinical trial, the data showed that atorvastatin treatment (40 mg/day) in addition to IFN- β -1a significantly increased the levels of serum ferric reducing antioxidant power and anti-inflammatory cytokines (IL-4, IL-10 and TGF- β) secretion and

decreased the levels of IL-17 and TNF- α cytokine secretion by PHA activated mononuclear cells of MS patient compared to IFN- β -1a monotherapy after a period of 18 months. This study showed that the levels of IFN- γ and NO were not affected by atorvastatin treatment.

Our data are consistent with the results of animal models and some clinical trial studies on MS patients. Recently, Weber *et al.* reported that atorvastatin treatment in IL-4 green fluorescent protein-enhanced transcript reporter mice, reduced secretion of proinflammatory cytokines (IFN- γ , IL-17, TNF- α and IL-12) (28). Neuhaus and co-workers observed decreased TNF- α secretion in simvastatin-treated activated peripheral blood mononuclear cells. They also showed no change in IFN- γ and IL-12 proinflammatory cytokine secretion (29). Moreover, Simvastatin inhibited secretion of Th17-polarizing cytokines and antigen presentation by DCs in MS patients (30). Other studies indicated that there is a reduction in the Treg number and the level of TGF- β in MS patients. There are only limited data suggesting that statins may promote expansion of Treg (31,32). In a previous study, we observed that the number of FoxP3+Treg cells was significantly increased after treatment with atorvastatin in an animal model of MS (11). In the present study, the level of TGF- β in the atorvastatin treatment group was significantly higher than the control group.

In addition, we observed that the lymphocyte proliferation decreased significantly compared to the baseline level after 18 months of treatment-MS patients with atorvastatin. Previous studies showed that statins inhibits lymphocyte proliferation *in vitro* (33). Weber *et al.* recently reported that 1 mg/kg oral atorvastatin treatment significantly shut down T-cell proliferation in EAE mice (28). Moreover, there are many reports that statins inhibit the proliferative activity of lymphocytes *in vitro* (10,29,34). However, these controversies in different studies might be due to differences in dose and type of statins (Atorvastatin, Simvastatin, Lovastatin), dose and type of interferon- β (1a or 1b), route of administration and genetic background of MS population. High quality randomized controlled trials are needed to provide better evidence for application of statins in MS treatment.

The limitations of our study can be summarized as:

- All MRI parameters could not be evaluated due to limited budget.
- Gd-Enhancing lesions are not a good outcome measurement when measured it once after 18 months but again we could not measure them in more intervals.
- As these patients had to receive medicine due to ethical issues we could not stop the treatment of a group as placebo control group.

In Conclusion, we found that although atorvastatin added-on to interferon therapy did not change the clinical course of the disease, atorvastatin decreased the secretion of IL-17 and TNF- α and increased the secretion of TH2 cytokines as well as TGF- β which may have beneficial therapeutic effect in MS treatment.

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