

Original Article

Evaluation of Rosuvastatin Therapy on *CYBB* Gene Expression in Patients with Multiple Sclerosis: An Uncontrolled Clinical Trial

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Abstract

Background and Objective: Multiple sclerosis (MS) is an autoimmune-mediated demyelinating disease. MS is a heterogeneous and complex disease with various degrees of progression and treatment responses to routine drugs. Recently, statins have been used for the treatment of MS due to their high efficacy and low side effects. This study aimed to investigate *CYBB* expression in MS patients undergoing rosuvastatin therapy.

Materials and Methods: This study was conducted on MS patients (n=24). Participants received 20 mg/day of rosuvastatin for 90 days. The Expanded Disability Status Scale (EDSS) and *CYBB* gene expression were measured before and after treatment with rosuvastatin. For gene expression measurement, total ribonucleic acid was isolated from MS patients' blood. After complementary DNA (cDNA) synthesis, the qualitative expression level of the *CYBB* gene was measured by real-time polymerase chain reaction technique (Roche LightCycler® 96). Additional information about patients was obtained and recorded in a checklist. Data were analyzed with statistical software GraphPad (version 6) and SPSS (version 23), and the significance level was $P<0.05$.

Results: Participants were 24 MS patients, including 5 (28.2%) males and 19 (79.2%) females, with an average age of 44.25 ± 11.12 years. No significant improvement was observed in the EDSS of patients following rosuvastatin therapy ($P>0.05$). Also, no significant correlation was observed between *CYBB* expression level and EDSS ($P>0.05$). *CYBB* was significantly upregulated in MS patients after rosuvastatin therapy ($P<0.001$), while no significant change was observed in the housekeeping gene expression ($P>0.05$).

Conclusions: The changes in *CYBB* gene expression occur earlier than any detectable clinical changes, such as those measured by EDSS.

Keywords: *CYBB*, Multiple sclerosis (MS), NADPH oxidase complex (NOX2), Rosuvastatin

Introduction

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) involving more than 900,000 people in the United States and about 2.8 million people worldwide [1-6]. MS prevalence is approximately 35.9 per 100,000 people and has increased since 2013 all over the world. The reported incidence of MS in 75 countries was 2.1 persons per 100,000 annually [6]. It mostly affects females and the average age at diagnosis is 32 years [6, 7].

MS is a chronic neurodegenerative and inflammatory disease that leads to progressive demyelination and axonal degeneration in the CNS (3-5, 8). MS is a heterogeneous disease with an almost unknown etiology. Both genetic (such as HLA-DRB1*15:01) and environmental factors (such as some viral infections, vitamin D, smoking, and obesity) play a role in its development [9, 10]. Clinical symptoms considerably vary based on the site and severity of neurologic lesions [11]. The diagnosis is based on clinical symptoms and magnetic resonance imaging (MRI) findings showing typical lesions of MS and/or cerebrospinal fluid-specific oligoclonal bands [12].

Available therapies for MS mainly inhibit auto-reactive T-cell activation and suppress inflammatory cell proliferation and migration to the CNS. Despite effective targeting of the above steps, the disease often progresses or relapses, which reflects the complex nature of the disease. Therefore, new therapies are required that more effectively prevent the progression and recurrence of the disease [13].

Statins are specific inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase enzyme, involved in the conversion of HMG-CoA to L-mevalonate, a crucial intermediate in the synthesis of cholesterol and nonsterol isoprenoids [14]. The anti-inflammatory, immunomodulatory, and neuroprotective effects of statins have been reported both in vitro and in experimental autoimmune encephalomyelitis (EAE) animal models, motivating several clinical trials with some surprising results [14]. Recent observations showed the protective role of statins in neuroprogenitor cells and enhanced remyelination in EAE models [13]. They also promote neuroregeneration and neurorepair in EAE, affect blood-brain barrier

integrity and inhibit the inflammatory cell infiltration into the CNS [15, 16]. Statins are different based on metabolism and lipophilicity. These vary in tissue penetration, safety, and drug interactions. Rosuvastatin, as a new generation of HMG-CoA reductase inhibitors, has unique pharmacokinetics and pharmacologic features. It has some advantages, including excellent low-density lipoprotein cholesterol (LDL-C) lowering effect, little extrahepatic tissue penetration, and minimal interactions with CYP3A4 [17]. MS disease has excessive heterogeneity concerning histopathological and radiological changes, clinical manifestations and progression, and also treatment response [18-23]. Therefore, it is crucial to find specific disease markers that enable easy diagnosis, prognosis, and treatment response evaluation. Currently, MRI findings and clinical characteristics are mainly used for monitoring patients and treatment response assessment [24]. However, it is impossible to quantify and standardize these features in individual patients; although it can be done in large patient groups [25, 26]. On the other hand, molecular biomarkers can be well quantified to complete clinical and MRI findings [27]. MS biomarkers usually have a causal role in the mechanism of disease development and progression [28]. The *CYBB* gene encodes the NADPH oxidase complex (NOX2) protein (other names: cytochrome b-245 and p91-phox), a part of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme complex [29]. NOX2 plays a crucial role in neurodegenerative disorders, including MS. The NADPH oxidases convert oxygen into superoxide anion radicals [30]. Increased NOX activity leads to the accumulation of reactive oxygen species (ROS) and oxidative stress, resulting in genomic instability. NOX also promotes the production of inflammatory cytokines and has a role in the pathogenesis of many inflammatory diseases [31]. Existing evidence shows that in addition to immunological factors, oxidative stress plays an important role in the etiology of MS and demyelination and axonal injury in EAE models and MS [32-34].

In the present study, the expression level of the *CYBB* gene was measured in MS patients before and after rosuvastatin therapy using real-time polymerase chain reaction (PCR) method to

determine the relationship between *CYBB* expression and treatment response.

Participants and Methods

Patients

This study was an uncontrolled clinical trial with a pre-post design performed in the Neshat clinic of Hamadan University of Medical Sciences, Hamadan, Iran. Patients aged 20-70 years with a definitive diagnosis of MS, which was confirmed by MRI, were included in the present study. Patients with other inflammatory diseases, cognitive and behavioral disorders, and poor cooperation were excluded from the study. Also, patients consuming antioxidant products during the last month, pregnant or lactating women, and patients with LDL-C >130 mg/dL were excluded from the study. Patients were withdrawn if intolerance to rosuvastatin occurred due to any side effects or if there was an increase in the patient’s disability. Finally, 24 patients were selected for this study using a convenience sampling method.

Treatments and Ethical Considerations

This research was approved by the Ethics Committee at Hamadan University of Medical Sciences, Hamadan, Iran (IR.UMSHA.REC.UMSHA.1400.697); all participants provided written informed consent after receiving the necessary information, and their data were kept confidential. Moreover, participation in this study was entirely voluntary, and no costs were incurred by the patients for taking part. This clinical trial has been documented and endorsed by the Iranian Registry of Clinical Trials (IRCT20210211050323N3).

Disability Measuring

An Expanded Disability Status Scale (EDSS) was used to measure MS-related disability. This scale is scored from 1 to 10 with 0.5 unit intervals; a higher score indicates a higher level of disability. A score of 0 indicates a normal neurological examination, whereas a score of 10 indicates death due to MS [35].

Ribonucleic Acid (RNA) Extraction and Complementary DNA (cDNA) Synthesis

Total RNA was extracted using RNX-Plus reagent (CinnaGen, Iran) from blood buffy coats taken from MS patients before and after rosuvastatin therapy according to the manufacturer's instructions. RNA quantity was measured using a NanoDrop spectrophotometer by measuring absorbance at 260 nm and 280 nm wavelengths. RNA quality was evaluated by running the total RNA on a 1% agarose gel. Complementary DNA (cDNA) was synthesized using the Parstous cDNA Synthesis Kit (Iran) with 1000 ng RNA according to the manufacturer's recommendations [36].

Real-Time Polymerase Chain Reaction (PCR)

The quantitative expression level of *CYBB* gene was measured by real-time PCR technique (Roche LightCycler® 96). cDNA (1 µL), forward (0.5 µL of 10 µM) and reverse (0.5 µL of 10 µM) gene-specific primers designed using AllelID software and analyzed with OligoAnalyzer (Table 1), were added to 12.5 µL SYBR Green II qPCR Mix (Sinaclone, Iran) and the final volume was increased to 25 µL using PCR-grade H₂O. Reactions were performed in 40 cycles (denaturation: 95°C for 30 s, annealing: 58-63°C for 30 s, extension: 72°C for 30 s), preceded by 15 minutes of pre-denaturation at 95°C. The specificity of PCR products was evaluated using melting peaks and electrophoresis on a 1% agarose gel. The *GAPDH* gene was used as the housekeeping gene and fold change was calculated using the 2^{-ΔΔCT} formula [36, 37].

Table 1. The Gene Specific Primers Used in Quantitative Gene Expression Measurement.

Gene Symbol	Primer Sequence (5'-3')
CYBB	Forward: TATGAGGTGGTGATGTTAGTGG
	Reverse: CAGATTGGTGGCGTTATTGC
GAPDH	Forward: AAGGCTGTGGGCAAGGTCATC
	Reverse: GCGTCAAAGGTGGAGGAGTGG

Statistical Analysis

The data were described by mean ± SD or frequency (%). Data were analyzed by one-way analysis of variance (ANOVA), independent *t-test*, paired *t-test*, and Pearson correlation tests using SPSS (version 23) and GraphPad Prism (version 6.0). The significance level was *P*<0.05.

Results

Patient Descriptions

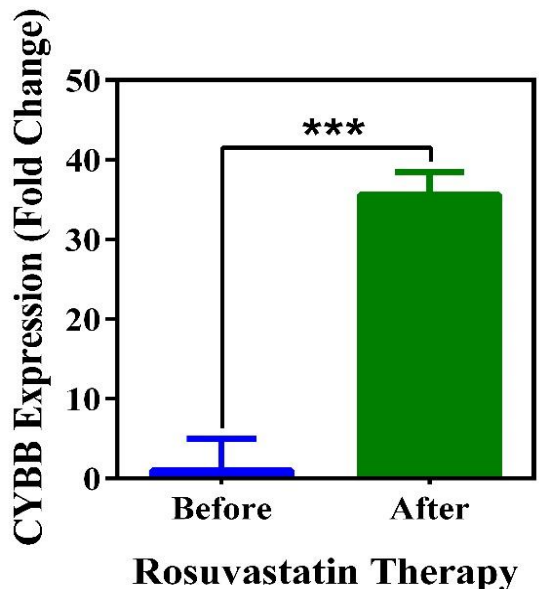
In this study, 24 patients, including 5 (28.2%) males and 19 (79.2%) females with an average age of 44.25 ± 11.12 years, were enrolled. Table 2 presents other patient characteristics.

Table 2. Description of Patients Enrolled in Study.

Patients Characteristics	
Sex, No. (%)	
Male	5 (28.2)
Female	19 (79.2)
Age (y) $\pm$ SD	44.25 ± 11.12
Weight (kg) $\pm$ SD	66.21 ± 11.90
Height (cm) $\pm$ SD	165.00 ± 7.19
BMI (kg/m ²) $\pm$ SD	24.37 ± 4.42
Prescribed Drugs, No. (%)	
Rituximab	12 (50.0)
Fingolimod	7 (29.2)
CinnoVex	2 (8.3)
Doxorubicin	1 (4.2)
Dalfyra	1 (4.2)
Others	1 (4.2)

CYBB Expression and Expanded Disability Status Scale (EDSS) Changes After Statin Therapy

Figure 1 shows that the CYBB gene was significantly upregulated in MS patients after



Rosuvastatin therapy (Table 3) ($P < 0.001$). However, no significant change was observed in the expression of the reference gene (*GAPDH*) following rosuvastatin therapy ($P > 0.05$) (Figure 2).

Figure 1. CYBB Expression Changes After Rosuvastatin Therapy. ***significant level at $P < 0.001$.

Variables	CYBB Gene Expression [Δ Ct]	
	Before R. T*	After R. T
EDSS Before R. T	** $r = 0.138$ $P = 0.540$	$r = 0.068$ $P = 0.763$
EDSS After R. T	$r = 0.206$ $P = 0.359$	$r = 0.123$ $P = 0.585$
Age	$r = -0.159$ $P = 0.479$	$r = 0.251$ $P = 0.260$
Weight	$r = 0.387$ $P = 0.075$	$r = 0.325$ $P = 0.140$
Height	$r = 0.013$ $P = 0.953$	$r = -0.310$ $P = 0.160$
BMI	$r = 0.370$ $P = 0.090$	$r = 0.442$ $P = 0.039$

Table 3. Relationship Between CYBB Gene Expression and Patients' Expanded Disability Status Scale (EDSS), Age, Weight, Height, and Body Mass Index (BMI).
*Rosuvastatin therapy, ** Pearson correlation coefficient.

On the other hand, a mild decrease was observed in EDSS scores (Table 4), but not significantly ($P > 0.05$) (Figure 3).

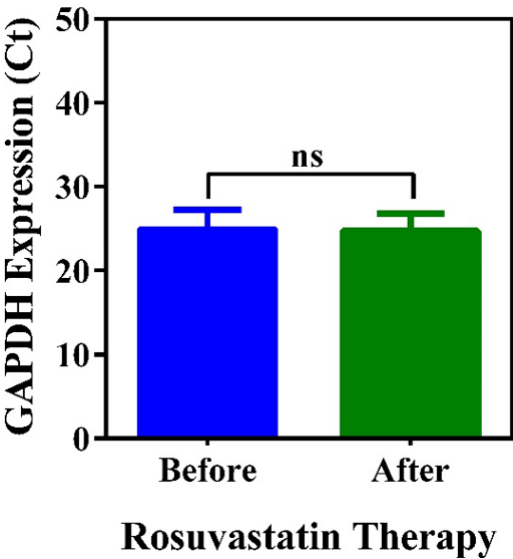


Figure 2. GAPDH Expression Changes After Rosuvastatin Therapy. ***significant level at $P < 0.001$; ns, not significant.

Table 4. Relationship Between Patients' Expanded Disability Status Scale (EDSS) Levels and Age, Weight, Height, and Body Mass Index (BMI).

Variables	EDSS	
	Before R. T*	After R. T
Age	** $r = 0.116$ $P = 0.588$	$r = 0.088$ $P = 0.683$
Weight	$r = -0.012$ $P = 0.954$	$r = 0.008$ $P = 0.972$
Height	$r = -0.011$ $P = 0.961$	$r = -0.098$ $P = 0.649$

BMI	$r = -0.020$	$r = 0.049$
	$P = 0.926$	$P = 0.821$

*Rosuvastatin therapy, ** Pearson correlation coefficient.

Relationship Between Patients' Demographic Data and *CYBB* Expression and Expanded Disability Status Scale (EDSS) Levels

No significant relationship was observed between EDSS and patient characteristics and between EDSS and *CYBB* gene expression (Table 5). No significant relationship was observed between *CYBB* gene expression levels and the characteristics of patients ($P>0.05$), except for body mass index (BMI). A positive and significant correlation was observed between BMI and *CYBB* gene expression ($P<0.05$).

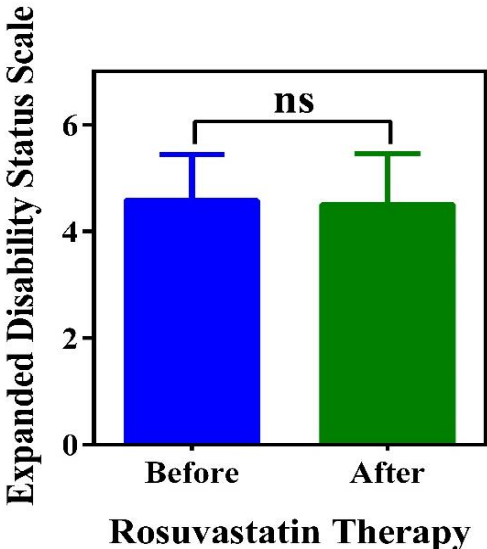


Figure 3. EDSS Changes After Rosuvastatin Therapy.

Table 5. Relationship of *CYBB* Gene Expression and Patients Expanded Disability Status Scale (EDSS) Level with Sex and Prescribed Drugs.

Variables	EDSS [±SD]		CYBB Gene Expression, ΔCt [±SD]	
	Before R. T*	After R. T	Before R. T	After R. T
Sex				
Male	4.20 (0.90)	4.10 (1.08)	5.97 (4.85)	1.40 (3.58)
Female	4.68 (0.83)	4.60 (0.92)	8.20 (3.72)	2.87 (2.57)
** <i>p</i>	0.269	0.303	0.283	0.315
Prescribed Drugs				
Dalfyra	4.50 (-)	4.50 (-)	8.70 (-)	-0.41 (-)
Doxorubicin	3.5 (-)	3.00 (-)	10.15 (-)	0.63 (-)
Fingolimod	4.57 (0.83)	4.85 (1.18)	9.65 (3.63)	1.64 (2.58)
Rituximab	4.66 (1.00)	4.41 (0.92)	6.87 (4.22)	3.27 (2.80)
CinnoVex	4.75 (0.35)	4.50 (0.00)	7.30 (5.02)	3.48 (5.38)

Others	4.50 (-)	4.50 (-)	2.38 (-)	2.82 (-)
# <i>p</i>	0.902	0.663	0.575	0.722

*Rosuvastatin therapy, independent *t* test, #One Way ANOVA test.

Discussion

MS is a chronic autoimmune condition characterized by demyelination of the CNS and is a heterogeneous disease showing varying degrees of disease progression [38]. The heterogeneous and complex nature of MS also leads to variable effectiveness of current therapies in different patients. Ineffective suppression of the inflammatory responses is often related to the rapid progression of the disease [39]. On the other hand, these therapies usually are associated with considerable side effects. Therefore, more effective neuroprotective and immunomodulatory drugs with a favorable efficacy profile are required [14]. Statins potentially have these properties and provide an opportunity for MS treatment because they have good safety and favorable immunomodulatory, anti-inflammatory, and neuroprotective effects, mainly by preventing isoprenylation of small GTP-binding proteins [14]. Continuous monitoring of drug therapy is an essential part of care of MS patients [40]. This study aimed to identify genes whose expression is correlated with treatment outcomes [41].

The *CYBB* gene encodes the NOX2 protein, which is a subunit of NOX [42]. The NADPH oxidase complex is involved in the production of ROS and leads to oxidative stress, which has been identified as a mediator of axonal injury and demyelination in animal models and also in MS patients [32, 33, 43]. In the study by Cardamone et al., *CYBB* expression has no significant difference between MS cases and healthy controls in both CD4⁺ and CD4⁻ cells. However, the *CYBB* gene was upregulated in relapsing-remitting MS patients when compared with healthy controls [34]. Gardon et al. reported that the *CYBB* gene was downregulated in animal models of MS and cerebral ischemia after treatment with phycocyanobilin [44]. Shahraz et al. showed that the *CYBB* gene contributes to neurodegeneration after repeated systemic challenge with lipopolysaccharides, in such a way that NOX2 knock-out mice showed attenuated neurodegeneration in comparison with control mice [45]. Ravelli et al. reported that NOX2 is involved in the pathogenesis of EAE and its deletion resulted in

disease improvement [46]. Fischer et al. indicated that NOX2 expression significantly increased in MS lesions compared to control white matter [47].

In the present study, the expression level of *CYBB* gene was significantly increased after 90 days of receiving rosuvastatin. However, no significant improvement was observed in the EDSS level following rosuvastatin therapy. Gene expression changes may occur earlier than clinical manifestations. In this regard, it has been shown that an ideal biomarker should be able to detect disease before the manifestation of clinical symptoms [48]. On the other hand, we did not observe any correlation between gene expression data and EDSS level. This can show that EDSS measurement is not enough for MS monitoring and other tools should be used, for example, imaging findings that are not present in our study and this is a limitation. This study investigated the changes in *CYBB* gene expression in MS patients after statin therapy for the first time. However, this investigation has some limitations, including a low number of samples, and the lack of imaging findings and other clinical information to evaluate the patients' response to treatment.

Conclusion

In this clinical trial, MS patients received rosuvastatin for 90 days, and their EDSS scores and *CYBB* gene expression were measured before and after the treatment period. Although no significant improvement was observed in the EDSS score of the patients, there was a significant increase in the expression level of the *CYBB* gene. We hypothesize that changes at the gene level occur earlier than the clinical manifestations. However, the current study was limited to a small number of samples. Therefore, we suggest that future studies should be conducted with a larger sample size.

Declarations

Ethical Approval and Consent to Participate

This study was approved by the Ethics Committee of Hamadan University of Medical Sciences, Hamadan, Iran (IR.UMSHA.REC.1400.697), and written informed consent was obtained from all patients. In this study, all experiments were performed in accordance with relevant guidelines

and regulations approved by the Ethics Committee of Hamadan University of Medical Sciences.

Consent for Publication

Not applicable.

Ethical Guidelines

This clinical trial was registered in the Iranian Registry of Clinical Trials (IRCT20210211050323N3).

Availability of Data and Material

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Funding

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Conflicts of Interest

The author(s) declared no conflicts of interest.

Authors' Contributions

The conception and design of the study: F.N and M.E; acquisition of data, analysis, and interpretation of data: F.N, R.S, A.S, and M.M; drafting of the article: R.S and F.N; revising the article critically for important intellectual content, final approval of the version to be submitted: F.N. All authors critically revised the manuscript and approved the final version.

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